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PRINCIPAL INVESTIGATOR: James C. Powers, Ph.D.

CONTRACTING ORGANIZATION: Georgia Technology Research Corporation
Georgia Institute of Technology
Atlanta, Georgia 30332

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James C. Powers, Ph.D.

7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES)
Georgia Technology Research Corporation
Georgia Institute of Technology
Atlanta, Georgia 30332

E-MAIL:
james.powers@chemistry.gatech.edu

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Sulfur mustard (SM) is a potent vesicant which penetrates the skin rapidly and causes extensive blistering. SM can alkylate DNA, RNA, and proteins which results in inflammation, tissue damage, and cell death. Many of the proteases released by SM exposure attack connective tissue proteins. It is likely that proteases are responsible for the formation of the fluid filled blisters which occur after SM exposure. Tissue homogenates harvested from the ears of mice or the backs of euthymic hairless mice exposed to SM were assayed for protease activities using chromogenic and fluorogenic substrates. SM exposed skin homogenates have higher protease activities than the control samples in both animal models. Three specific protease inhibitors reduce the protease activities of the exposed and control samples in both animal models. Skin tissue homogenates pretreated with anti-inflammatory drugs, protease inhibitors, or a combination of the two prior to the SM exposure have lower protease activities than untreated samples. Protease assays are useful in measuring the extent and progress of SM induced vesication and can be used to measure the effectiveness of drug treatments. Protease inhibitors and antiinflammatory drugs alone or in combination may have therapeutic use in reducing tissue injury caused by SM exposure.

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James C. Powers 10/31/99

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SUMMARY AND CONCLUSIONS

- **Proteolytic Enzymes (Proteases) Are Markers of SM Induced Vesication.**
  - Skin tissue homogenates from the mouse ear vesicant model or euthymic hairless mice exposed to SM were assayed for serine and cysteine protease activities using sensitive chromogenic and fluorogenic protease substrates.
  - Skin homogenates exposed to SM had higher protease activities than control samples in the mouse ear vesicant model and the euthymic hairless mouse model.
  - Proteolytic enzyme assays are useful for measuring the extent and progress of SM induced vesication.
- **Protease Assays Can Be Used to Follow the Course and Effectiveness of Drug Treatments.**
  - Specific protease inhibitors reduced the protease activities of the exposed and control samples in both animal models.
  - Skin tissue homogenates from animals pretreated with anti-inflammatory drugs, protease inhibitors or combination of both prior to exposure to SM had lower protease activities.
  - Protease assays can be used to evaluate drug therapy and should be useful for screening new drug candidates.
  - Antiinflammatory drugs should have therapeutic use in reducing tissue injury caused by SM exposure.
  - Protease inhibitors should also have therapeutic utility in treatment of SM induced vesication.

ABSTRACT

Biochemical Markers and Synthetic Protease Inhibitors in Animal Models of Sulfur Mustard Vesication

Keywords: Antiinflammatory Drugs, Antivesicants, Chromogenic Substrates, Cysteine Proteases, Fluorogenic Substrates, Metalloproteinases, Protease Inhibitors, Serine Proteases, Sulfur Mustard.

Sulfur mustard (bis-(2-chloroethyl)sulfide, HD or SM) is a potent vesicant which penetrates the skin rapidly and causes extensive blistering. Currently, there is no effective antidote or pretreatment for SM induced cutaneous injury. Although the molecular mechanisms for SM induced injury are not clear, SM can alkylate DNA, RNA, and proteins which results in inflammation, tissue damage, and cell death. Proteolytic enzymes (proteases) such as elastase, chymases, tryptases, matrix metalloproteases, and cathepsins are released or induced during this process. Many of the proteases released by SM exposure attack connective tissue proteins such as collagen, elastin and proteoglycan. It is likely that proteases are responsible for the formation of the fluid filled blisters which occur after SM exposure and are also important mediators in the inflammatory process.

Proteolytic enzyme activity was found in SM mustard induced blistering in two animal models, the mouse ear vesicant model and the euthymic hairless mouse model. Skin tissue homogenates from these animals exposed to SM were assayed for serine and cysteine protease activities using chromogenic thioester and fluorogenic AMC (AMC = 7-amino-4-methylcoumarin) substrates. The samples from the mouse ear vesicant model include three groups of skin homogenates: The first group of skin homogenates was obtained from the mouse ear without pretreatment with any drug prior to SM exposure. The second group of skin homogenates was obtained from animals pretreated with an anti-inflammatory drug on the skin prior to the SM exposure. The third group of samples was obtained from animals pretreated with a protease inhibitor or a combination of an anti-inflammatory drug and a protease inhibitor on the skin prior to the SM exposure. The samples from euthymic hairless mouse model are skin homogenates from the back of hairless mice without pretreatment with a drug prior to SM exposure.

In the first group of samples from the mouse ear vesicant model, exposed tissue samples were obtained from the ears of mice (n = 6) harvested 3, 6, 12, and 24 h after exposure to SM in CH_2Cl_2 on their right ear. Control samples treated only with dichloromethane vehicle were obtained from the left ear of the same animal. These control samples can be used as a marker for systemic inflammation. Naive controls were obtained from both

left and right ears of animals which received no SM (n = 3). Naive controls are indicative of the background levels of proteases in the samples. Skin homogenates from mice exposed to SM have substantially elevated levels of the proteolytic enzymes elastase, tryptase, and calpain 24 h after SM exposure compared to control samples. Other proteolytic enzymes such cathepsin B, cathepsin H, chymase, plasmin, and thrombin were found in mouse skin from both SM exposed and unexposed animals. Clearly, several proteolytic enzymes are excellent markers of SM induced vesication.

Synthetic protease inhibitors can abolish the proteolytic activity found in skin homogenates from the mouse ear exposed to SM. Three synthetic inhibitors, one Val phosphonate (an elastase inhibitor), one amidine-containing phosphonate (inhibitor of tryptases), and one ketoamide (calpain and cathepsin B inhibitor), were used to inhibit the proteolytic activities of the tissue homogenates. The Val phosphonate effectively inhibited the elastase activities of both exposed and control samples. The amidine phosphonate inhibited the tryptase activity of exposed sample more potently than control samples. This may indicate that a unique tryptase is expressed or released after SM exposure. The ketoamide completely abolished the cathepsin B activity of both exposed and control samples and also partially inhibited the calpain activity of exposed samples.

In the second group of animals from the mouse ear vesicant model, tissue homogenates were harvested from the skin of the mouse ear exposed to SM after pretreatment with ethanol (positive control) or an anti-inflammatory drug (olvanil, retro-olvanil, and indomethacin) for 15 min. The SM exposed tissue samples were obtained from animals (n = 10) at 6, 12, 24, 48 and 72 h postexposure from the right ear, while control samples treated only with dichloromethane were obtained from left ear. Naive controls (left and right ear) were obtained from animals which received no SM treatment (n = 10). The pretreatment of exposed samples with these three drugs had some effects on various protease activities at different harvest time (12, 24, or 48 h). At 6 h postexposure, only slight changes in enzyme activities in samples after being pretreated with ethanol, olvanil or indomethacin were observed. At 48 h postexposure, skin homogenates from animals pretreated with olvanil or indomethacin in ethanol before SM exposure had lower elastase, tryptase, calpain, and chymase activities than those pretreated only with ethanol. Samples pretreated with retro-olvanil had lower tryptase activity when compared to samples only pretreated with ethanol. Clearly, anti-inflammatory drugs reduce proteolytic enzyme activity in the mouse ear vesicant model.

Samples pretreated with ethanol and harvested at 72 h postexposure had lower protease activities than the samples pretreated with ethanol and harvested at 48 h postexposure. This is due to the fact that samples harvested at 72 h postexposure were stored in the freezer for many months and lost some enzyme

activities during this period. Future proteolytic enzyme assays should be carried out shortly after the samples are harvested.

In the third group of samples from the mouse ear vesicant model, tissue homogenates were harvested from the skin of the mouse ear exposed to SM after pretreatment with ethanol (positive control) or protease inhibitors (3-chloroisocoumarin, Suc-Val-Pro-$Phe^{P}(OPh)_2$, or 3-chloroisocoumarin + olvanil) for 15 min. The SM exposed tissue samples were obtained from animals (n = 10) at 6, 12, 24, 48 and 72 h postexposure from the right ear, while control samples treated only with dichloromethane were obtained from the left ear. Naive controls (left and right ear) were obtained from animals which received no SM treatment (n = 10). The pretreatment of exposed samples with these three drugs reduced protease activities at various harvest time (12, 24, 48 or 72 h). At 72 h postexposure, samples pretreated with 3-chloroisocoumarin or (3-chloroisocoumarin + olvanil) had lower elastase, chymase, tryptase, and calpain activities when compared to samples only pretreated with ethanol; Samples pretreated with phosphonate had lower chymase and cathepsin B activities when compared to samples only pretreated with ethanol. Only slight changes in enzyme activities in samples harvested at 6, and 12 h postexposure after being pretreated with ethanol, 3-chloroisocoumarin, or (3-chloroisocoumarin + olvanil) were observed. The isocoumarin pretreatment did not change the enzyme activities of cathepsin B and cathepsin H in the exposed samples at various postexposure times and the phosphonate pretreatment did not change the tryptase activity of the exposed samples. Clearly, protease inhibitors also reduce proteolytic enzyme activity in mouse ear vesicant model.

In the euthymic hairless mouse model, tissue homogenates were prepared from either sham-treated or SM exposed skin from the back of mice. The tissue samples were obtained from animals (n = 8) at 2, 6, and 24 h postexposure. Each animal has four exposure sites, two controls and two SM-exposed sites. At 6 h postexposure, exposed samples had higher thrombin, calpain, and cathepsin H activities when compared to the control samples. At 24 h postexposure, exposed samples had higher elastase and plasmin activities when compared to the control samples. No difference in tryptase, chymase, and cathepsin B activities was detected between the exposed and control samples. The same protease inhibitors were tested on the proteolytic activities of the tissue homogenates from the euthymic mouse. The Val phosphonate (elastase inhibitor) completely inhibited the elastase activity of exposed and control samples, and the ketoamide inhibitor abolished the cathepsin B and calpain activities of exposed and control samples. However, the amidine phosphonate was only partially effective at inhibiting the tryptase-like activity in the exposed and control samples.

Our data demonstrate that proteases are involved in SM skin injury. Anti-inflammatory drugs or protease inhibitors reduce proteolytic enzyme activity which indicates that protease assays can be used to follow the course of treatment following SM induced

injury and can be used for the screening of new drug candidates to SM induced vesication. Specific protease inhibitors, anti-inflammatory drugs, and a combination of the two should have therapeutic utility in reducing or eliminating tissue injury caused by SM cutaneous exposure.

INTRODUCTION

Sulfur mustard (bis-(2-chloroethyl)sulfide, HD or SM) is a potent vesicant which penetrates the skin rapidly and causes erythema, edema, necrosis and extensive blistering. Currently, there is no effective antidote or pretreatment for SM induced cutaneous injury. Although the molecular mechanisms for SM induced injury are not clear, SM can alkylate DNA, RNA, and proteins and causes inflammation, tissue damage, and cell death.[1] Papirmeister has suggested that the alkylated purine bases in DNA are unstable and undergo both spontaneous and enzymatic depurination.[2] The alkylation of DNA can result in DNA strand breakdown, and activation of nucleases and other DNA repair mechanisms. As a result, poly(ADP-ribose)polymerase is activated which in turn leads to interference in energy metabolism.[3] Other mechanisms of SM cytotoxicity are also hypothesized, and it is likely that multiple, interacting pathways are simultaneously involved in SM toxicity.

Proteolytic enzymes are released or induced as a result of cell death and tissue damage. A variety of mechanisms may be responsible for the presence of these powerful digestive enzymes. The breakup of a variety of cells including mast cells and neutrophils would result in the release of enzymes such as elastase, chymases, various tryptases (trypsin-like enzymes), matrix metalloproteases (MMPs), and cathepsins. Tissue inflammation attracts neutrophils and macrophages which release proteolytic enzymes as part of the inflammatory process and can cause activation of the complement system. The presence of chemically modified proteins formed by SM alkylation would stimulate intracellular proteases such as the multicatalytic proteases which are normally involved in removing incompletely synthesized proteins or defective proteins produced by oxidation or other chemical modifications. Many of the proteases released upon exposure to SM attack connective tissue proteins. It is likely that digestion of connective tissue proteins by proteases is responsible for the vesication observed upon exposure to SM.

Proteases are normally controlled by natural plasma protein inhibitors such as α_1-protease inhibitor and α_1-antichymotrypsin. If this protease inhibitor screen is destroyed, tissue destruction results. Several of the plasma serpins (serine protease inhibitors) including α_1-protease inhibitor and α_1-antichymotrypsin have essential methionine residues and are susceptible to inactivation by oxidizing agents and alkylating agents. A single dose of sulfur mustard in the mouse brain has been shown to cause a burst of oxidants.[4] Thus, the serpin screen could be removed either directly by sulfur mustard alkylation or indirectly by oxidation. As a result, sulfur mustard exposure results in the release of powerful proteolytic enzymes and the destruction of the protease inhibitor screen which would normally protect tissue from proteolysis.

Previous studies have demonstrated that proteases such as elastase and tryptase activities are increased in sulfur mustard-exposed peripheral blood lymphocytes[5] and hairless guinea pig skin homogenates.[6,7] The protease activities are inhibited by several protease inhibitors.[6,8] These results indicate that proteases may play a role in sulfur mustard induced inflammation and vesication.

The mouse ear vesicant model has been used to study chemical-induced cutaneous inflammation using edema, histopathology, biochemical and inflammatory mediators as endpoint.[9,10,11] In this study we have assayed skin homogenates from mouse ear and euthymic hairless mice after exposure to SM for several protease activities using sensitive chromogenic and fluorogenic substrates. We have shown that several protease activities of exposed homogenates are increased due to SM exposure in both models. We also investigated the inhibitory effects of three synthetic inhibitors on the proteolytic activity of the homogenates of both models. In the mouse ear vesicant model, pretreatment of mouse ear with anti-inflammatory drugs or protease inhibitors prior to the SM exposure results in a decrease in several enzyme activities in the skin homogenates harvested at 24, 48, or 72 h postexposure. These results demonstrate the enhancement of several proteolytic activities during SM induced tissue injury. Thus, effective protease inhibitors should be useful for studying the cytotoxicity mechanisms of sulfur mustard and may have therapeutic use in reducing tissue injury caused by sulfur mustard exposure.

Statement of Work

I. Develop new biochemical markers for sulfur mustard (SM) induced toxicity.

We proposed to assay skin homogenates from mice exposed to sulfur mustard for protease activities using sensitive chromogenic and fluorogenic substrates. The proteolytic activity would be correlated with the severity of blistering and the inflammatory response following SM exposure. The proteolytic enzyme activities were studied as a function of time of harvesting the samples.

This was a collaborative research project with Dr. Michael C. Babin of USAMRICD and he supplied the skin homogenates of mouse ear vesicant model used in the research. Samples of euthymic hairless mouse model were obtained from the Battelle Research Corp.

II. Assay the inhibitory potency of synthetic protease inhibitors toward the proteolytic activities present in skin homogenates from mice exposed to SM.

We proposed to determine the most potent inhibitors for the various proteolytic enzyme activities which were present in skin homogenates exposed to SM. We used samples of inhibitors available in the laboratory of the PI and included inhibitors previously submitted to the U.S. Army for testing in the vesicant program.

III. Correlate the activity of synthetic protease inhibitors *in vitro* with their *in vivo* activity.

The protease inhibitors found to decrease or eliminate the inflammatory response in SM exposed animals were studied for their ability to inhibit various proteases *in vitro*.

EXPERIMENTAL METHODS

Materials. HEPES[a] and PIPES were purchased from Research Organics, Inc., Cleveland, OH. DTNB, EDTA, DTT, and Brij 35 were purchased from Aldrich Chemical Co., Milwaukee, WI. The fluorogenic substrates Z-Arg-Arg-AMC, Arg-AMC, Z-Arg-AMC, Tos-Gly-Pro-Arg-AMC, Suc-Ala-Ala-Pro-Phe-AMC, Suc-Ala-Phe-Lys-AMC, Suc-Leu-Tyr-AMC and Mca-Pro-Leu-Gly-Leu-Dpa-Ala-Arg-NH_2 were obtained from Bachem Bioscience Inc., King of Prussia, PA. The chromogenic substrates Z-Arg-SBzl,[12] Suc-Ala-Ala-Pro-Phe-SBzl,[13] Suc-Phe-Leu-Phe-SBzl[13] and Boc-Ala-Ala-Ala-SBzl,[14] and the inhibitors Boc-Val-Pro-Val^P(OPh)$_2$,[15] Z-Leu-Abu-CONH-$(CH_2)_3$-4-Mpl,[16] and *o*-phenoxybenzoyl-Pro-4-AmPhGly^P(OPh)$_2$ [17] were synthesized in the laboratory of the PI by previously described methods.

Animals.

Mouse Ear Vesicant Model. Male CD1 mice (Charles River Laboratories, Raleigh, NC) 25-35 g, were maintained under an AAALAC program.

Euthymic Hairless Mouse Model. Euthymic hairless mice were maintained under Battelle's Institutional Animal Care and Use Committee (IACUC) for the conduct of Task Order No. 95-41.

SM Exposure.

Mouse Ear Vesicant Model. In the first group, a group of mice, 6 per time point were weighed, anesthetized, and exposed to SM according to a previously described method.[11] The right ears of mice were exposed to 5 µL of SM solution (32 mg/mL SM in CH_2Cl_2, 0.16 mg/ear), the left ears received 5 µL of CH_2Cl_2, and naive animals received nothing. After exposure to SM, animals were maintained for 3, 6, 12, or 24 h and then euthanized individually.

In the second group, a group of mice (n =10) were treated with anti-inflammatory drugs before exposure to SM. The right ear of each animal was pretreated with ethanol (10 µL), olvanil (10 µL of 25 mg/mL, 0.25 mg/ear), retro-olvanil (10 µL of 25 mg, 0.25 mg/ear), indomethacin (20 µL of 50 mg/mL, 1.0 mg/ear) for 15 min and then exposed to SM solution, while the left ear received a pretreatment of CH_2Cl_2 (5 µL). The naive animals did not receive any treatment.

In the third group, a group of mice (n = 10) were treated with protease inhibitors before exposure to SM. Each animal was pretreated with ethanol (10 µL), 3-chloroisocoumarin (30 µL of 20 mg/mL, 0.6 mg/ear), Suc-Val-Pro-Phe^P(OPh)$_2$ (10 µL of 100 mg/mL, 1.0

mg/ear), or 3-chloroisocoumarin (30 µL of 20 mg/mL, 0.6 mg/ear) plus olvanil (10 µL of 25 mg/mL, 0.25 mg/ear) for 15 min on the right ear and then exposed to SM solution, while the left ear received a pretreatment of CH_2Cl_2 (5 µL). The naive animals did not receive any treatment.

Euthymic Hairless Mouse Model. Euthymic hairless mice were exposed on the dorsal surface, with four exposure sites per animal. Two sites per animal were controls and two were SM-exposed. The skin specimens used for homogenate preparation were full-thickness, approximately 12-mm diameter punches taken from euthanized animals at 2, 6, or 24 h following SM exposure. Upon collection, each sample was snap frozen in liquid nitrogen and stored at -70 °C until processed.

Skin Homogenates of Mouse Ear Vesicant Model.

Mouse Ear with No Pretreatment. At 3, 6, 12, and 24 h post SM exposure, skin tissue was collected and kept in a -70 °C freezer. Frozen samples were ground using a BioPulverizer and liquid nitrogen. Homogenates were prepared in 1.5 mL of phosphate buffered saline (PBS) and centrifuged at 2500 rpm for 10 min at 0 °C. The supernatant was aspirated and placed in a vial as the first suspension. Next the pellet from the sample was resuspended in 1.5 mL PBS and centrifuged again. The supernatant was aspirated and placed in another vial as the second suspension. The same procedure was used to obtain the third suspension. At each time point, 6 first suspensions, 2 second, and 2 third suspensions were obtained from control and exposed samples, while 3 first suspensions, and 2 second suspensions were obtained from right and left ears of naive animals.

Mouse Ear with Pretreatment. At 6, 12, 24, 48 and 72 h post SM exposure, skin tissue from pretreated mouse ear was collected and kept in a -70 °C freezer. Frozen samples were ground using a BioPulverizer and liquid nitrogen. Homogenates were prepared in 2.0 mL of phosphate buffered saline (PBS) and centrifuged at 2500 rpm for 10 min at 0 °C. The supernatant was aspirated and split into two vials. One vial was used for testing enzyme activities.

Skin Homogenates of Euthymic Hairless Mouse Model. Frozen specimens were crushed using a BioPulverizer (Daigger Scientific). The crushed material was weighed and resuspended in phosphate buffered saline (PBS). Suspensions were centrifuged at 50,000 x g for 30 min, and the supernatant drawn off for soluble biomarker analysis. All samples were stored at -70 °C.

Protease Substrate Assays. Elastase, chymase, tryptase, thrombin, and plasmin activities of mouse skin homogenates were measured in 0.1 M HEPES, 0.5 M NaCl, pH 7.5 and at 23 °C. An aliquot of homogenate (10 or 25 µL) was added to an assay mixture of 0.2 mL of buffer, 10 µL of 5 mM substrate in DMSO (0.21 mM Boc-Ala-Ala-Ala-SBzl for elastase, 0.21 mM Suc-Ala-Ala-Pro-Phe-SBzl or Suc-Phe-Leu-Phe-SBzl for chymase, and 0.21 mM Z-Arg-SBzl for tryptase) and 10 µL of 5 mM DTNB in DMSO. The hydrolysis rates were monitored spectrophotometrically at 405 nm for 5-60 min with

a Thermomax microplate reader (Molecular Devices, CA). Chymase, tryptase, thrombin and plasmin activities were also measured using fluorogenic substrates Suc-Ala-Ala-Pro-Phe-AMC (0.21 mM), Z-Arg-AMC (0.21 mM), Tos-Gly-Pro-Arg-AMC (0.21 mM), and Suc-Ala-Phe-Lys-AMC (0.21 mM). The hydrolysis rates were monitored by the change in fluorescence (λ_{ex} = 360 nm, λ_{em} = 465 nm) for 1 h using a Tecan Spectrafluor plate reader.

Cathepsin B activity was measured in 0.1 M KH_2PO_4, 1.25 mM EDTA, and 0.01% Brij 35 at pH 6.0.[18] Cathepsin H activity was measured in 50 mM Pipes, 2.5 mM EDTA, 0.01% Brij 35, and 1 mM DTT at pH 6.8.[18] Calpain activity was measured in 50 mM HEPES, 10 mM cysteine, and 5 mM $CaCl_2$ at pH 7.5.[19] For cathepsin B, cathepsin H and calpain activities, 10 µL of homogenate was added to 0.2 mL of buffer containing fluorogenic substrate (0.49 mM Z-Arg-Arg-AMC for cathepsin B, 0.11 mM Arg-AMC for cathepsin H, and 0.62 mM Suc-Leu-Tyr-AMC for calpain) and the hydrolysis rates were measured using the change in fluorescence (λ_{ex} = 360 nm, λ_{em} = 465 nm) for 1 h using a Tecan Spectrafluor plate reader.

Matrix Metalloprotease activity was measured in 50 mM Tris, 0.15 M NaCl, 10 mM $CaCl_2$, and 0.05% Brij 35, pH 7.5 or in 50 mM NaAc, 0.15 M NaCl, 10 mM $CaCl_2$, and 0.05% Brij 35 pH 6.0 and at 23 °C. Homogenates (25 µL) were added to 0.475 mL of buffer containing the substrate Mca-Pro-Leu-Gly-Leu-Dpa-Ala-Arg-NH_2 (6 µL)[20] and the hydrolysis rates were measured using the change in fluorescence (λ_{ex} = 328 nm, λ_{em} = 393 nm) for 5 min in a Spex fluorometer.

Inhibitor Studies. Inhibition of elastase and tryptase activities by two phosphonate inhibitors was monitored using an incubation method. A 25 µL aliquot of homogenate (12 and 24 h postexposure) was incubated with 10 µL of an inhibitor solution (0.043 mM Boc-Val-Pro-Val^{P}(OPh)$_2$) in 200 µL of buffer for 1 h. A 10 µL aliquot of 5 mM Boc-Ala-Ala-Ala-SBzl and 10 µL of 5 mM DTNB were then added and the hydrolysis rates were monitored at 405 nm for 1 h. Inhibition of tryptase activity by *o*-phenoxybenzoyl-Pro-4-AmPhGly^{P}(OPh)$_2$ (0.21 mM) was measured under similar conditions except that 10 µL of 5 mM Z-Arg-SBzl was used as a substrate.

For cathepsin B and calpain, 10 µL of 2.5 mM Z-Leu-Abu-CONH-$(CH_2)_3$-4-Mpl and 25 µL of homogenate (3 and 6 h postexposure for cat B; 12 and 24 h postexposure for calpain) were added to 0.2 mL of buffer containing substrate (0.49 mM Z-Arg-Arg-AMC for cathepsin B and 0.62 mM Suc-Leu-Tyr-AMC for calpain) and the hydrolysis rates were monitored spectrofluorometrically (λ_{ex} = 360 nm, λ_{em} = 465 nm) for 1 h. Percent inhibition is the average of 6 control samples or 6 exposed samples.

RESULTS

I. Mouse Ear Vesicant Model

1. Protease Activity of Skin Homogenates from Mouse Ear without Any Pretreatment.

Proteolytic enzyme activities were found in SM induced blistering of mouse ear vesicant model. Skin tissue homogenates from these animals exposed to SM were assayed for serine and cysteine protease activities using chromogenic thioester and fluorogenic AMC (AMC = 7-amino-4-methylcoumarin) substrates. Skin homogenates from mice exposed to SM have substantially elevated levels of the proteolytic enzyme activities when compared to control samples. Clearly, several proteolytic enzymes are excellent markers of SM induced vesication.

Most of the 120 mouse skin homogenates were tested with 10 different protease substrates (Table I). The thioesters are sensitive chromogenic substrates for serine proteases and the aminomethylcoumarin derivatives (AMC) are sensitive fluorogenic substrates for serine and cysteine proteases. Four of the AMC derivatives are for tryptase, thrombin, plasmin, and chymase, while the remainders are specific for cysteine proteases. The methoxycoumarin (Mca)-containing peptide is a sensitive substrate for several matrix metalloproteases.

The enzymatic activity of all samples was measured with all 10 substrates except for the Mca-containing peptide. Metalloprotease activity was only measured with samples at 24 h postexposure due to the limited amount of available Mca substrate. Data from the first suspensions are shown in Table II, data for the second and third suspensions are shown in Table 1 and 2 in the Appendices. Generally, the first suspensions have higher enzymatic activities than those in the second and third suspensions. It is probably not worthwhile to examine second and higher suspensions since the majority of enzymatic activity is in the first suspensions. Each value except for the metalloprotease is the average of 12 data points from 6 samples at the same site on 6 different animals. The value for the naive control is the average of 12 data points from 6 samples at both sites on 3 different animals. The standard error of each value is shown in the tables.

Table I. Chromogenic and Fluorogenic Substrates Used to Evaluate Proteolytic Enzyme Activities in the Mouse Ear Vesicant Model.

Substrate	Protease
Boc-Ala-Ala-Ala-SBzl	elastase
Z-Arg-SBzl	tryptase
Z-Arg-AMC	tryptase
Tos-Gly-Pro-Arg-AMC	thrombin
Suc-Ala-Phe-Lys-AMC	plasmin
Suc-Ala-Ala-Pro-Phe-AMC	chymase
Suc-Ala-Ala-Pro-Phe-SBzl	chymase
Z-Arg-Arg-AMC	cathepsin B
Arg-AMC	cathepsin H
Suc-Leu-Tyr-AMC	calpain
Mca-Pro-Leu-Gly-Leu-Dpa-Ala-Arg-NH_2	matrix metalloproteases

Serine and Cysteine Protease Activity. Skin samples obtained from SM exposed animals harvested at 12 and 24 h postexposure had higher elastase activity than control samples (Fig. 1). Exposed samples showed a 2-19 fold increase in elastase activity when compared to control samples. Elastase activities of both exposed and control samples increased when the amounts of homogenate (10 to 50 µL) used in the assays was increased (Fig. 2). The dose-response curve indicates the elastase activities of these samples are real. For tryptase (Fig. 3 & 4), thrombin (Fig. 5), plasmin (Fig. 6) and calpain (Fig. 7) activities, only exposed samples at 24 h postexposure showed higher activities when compared to control samples. Both Z-Arg-SBzl and Z-Arg-AMC were used to measure tryptase activity and gave similar results. For elastase, tryptase and calpain activities, no differences from controls were observed with samples from SM exposed skin obtained at 3 and 6 h postexposure. Generally, both unexposed and SM exposed skin had distinct cathepsin B (Fig. 8) and cathepsin H (Fig. 9) activities and some chymase activity (Fig. 10).

Metalloprotease Activity. The metalloprotease activities of SM exposed samples at 24 h postexposure had higher enzyme activities than control samples at pH 6.0 (Table II). The enzyme activities of both exposed and control samples increased 2 times when homogenates used in the assay were increased from 25 µL to 100 µL (Fig. 11). However, small differences were observed between control and exposed samples at pH 7.5.

Table II. Enzymatic Activities of Mouse Ear Skin Homogenates (First Suspensions).[a]

Hours Postexposure	Enzyme Activity (mOD/min or Fl/min)		
	Exposed Samples	Control Samples	Naive Control
Elastase			
3	0.102 ± 0.021	0.040 ± 0.020	0.130 ± 0.038
6	0.325 ± 0.287	-0.157 ± 0.195	0.117 ± 0.033
12	0.398 ± 0.141	0.052 ± 0.025	0.054 ± 0.069
24	0.658 ± 0.121	0.033 ± 0.022	0.041 ± 0.013
Tryptase			
3	0.741 ± 0.456	0.631 ± 0.447	0.189 ± 0.358
6	2.320 ± 0.653	2.135 ± 0.578	0.828 ± 0.341
12	2.426 ± 0.646	1.819 ± 0.404	1.062 ± 0.272
24	6.582 ± 1.045	2.043 ± 0.364	2.333 ± 1.071
Chymase			
3	2.739 ± 0.636	2.140 ± 0.283	2.804 ± 0.504
6	2.717 ± 0.182	2.285 ± 0.561	2.410 ± 0.258
12	2.474 ± 0.311	2.283 ± 0.450	2.898 ± 0.500
24	2.921 ± 0.093	3.208 ± 0.547	1.617 ± 0.430
Plasmin			
3	10.68 ± 4.26	2.534 ± 1.326	10.33 ± 2.809
6	0.56 ± 0.76	2.499 ± 0.903	-2.92 ± 0.649
12	1.19 ± 1.60	3.678 ± 3.489	-2.79 ± 0.382
24	10.26 ± 3.25	2.082 ± 0.591	0.334 ± 1.582
Thrombin			
3	32.71 ± 6.99	21.57 ± 5.259	87.96 ± 33.47
6	66.22 ± 26.97	35.62 ± 10.02	38.89 ± 18.72
12	63.75 ± 14.72	49.26 ± 17.91	59.33 ± 11.43
24	117.70 ± 22.14	78.72 ± 25.13	33.44 ± 12.88
Cathepsin B			
3	344.8 ± 26.59	275.7 ± 28.31	371.4 ± 28.83
6	381.3 ± 48.11	239.5 ± 43.12	312.6 ± 54.81
12	358.0 ± 43.25	315.0 ± 45.69	346.0 ± 44.37
24	241.9 ± 26.78	251.5 ± 42.63	222.4 ± 40.11
Calpain			
3	3.028 ± 1.131	1.731 ± 0.367	2.748 ± 0.258
6	4.432 ± 0.472	2.583 ± 0.206	4.539 ± 0.469
12	5.614 ± 1.129	2.716 ± 0.194	3.748 ± 0.694
24	6.235 ± 1.346	2.334 ± 0.393	2.051 ± 0.287
Cathepsin H			
3	191.2 ± 23.67	147.8 ± 16.55	120.8 ± 25.93
6	183.8 ± 22.55	164.7 ± 28.53	138.4 ± 9.056
12	202.6 ± 20.45	166.3 ± 28.67	271.8 ± 52.35
24	259.0 ± 25.49	232.3 ± 40.75	272.3 ± 60.23
Metalloproteases			
24 (pH 7.5)	13.07 ± 4.577	22.90 ± 11.90	16.53 ± 3.210
24 (pH 6.0)	28.27 ± 8.423	12.76 ± 2.920	15.51 ± 1.830

[a]Conditions are described in Materials and Methods.

Inhibition of Protease Activity. Three synthetic protease inhibitors (structures shown in Fig. 12) were used to inhibit the proteolytic activities of mouse ear skin homogenates. These inhibitors include the specific elastase inhibitor [Boc-Val-Pro-Val^{P}(OPh)$_2$], the tryptase inhibitor [*o*-phenoxybenzoyl-Pro-4-AmPhGly^{P}(OPh)$_2$], and the calpain and cathepsin B inhibitor [Z-Leu-Abu-CONH-(CH$_2$)$_3$-4-Mpl]. These inhibitors were tested on 6 control samples and 6 samples at 12 and 24 h postexposure. For cathepsin B, samples harvested at 3 and 6 h postexposure were used.

Table III. Inhibition of protease activities in homogenates of mouse ear skin exposed to SM.

Inhibitors	Samples (postexposure time)	Inh. Conc (μM)	Enz. Act. (no I) mOD/min	% Inh.
Elastase Activity				
Boc-Val-ProVal^{P}(OPh)$_2$	Naive (12 h)	43	0.99	89
	Control (12 h)	43	0.58	89
	Exposed (12 h)	43	8.32	91
	Naive (24 h)	43	0.86	92
	Control (24 h)	43	0.72	86
	Exposed (24 h)	43	26.0	86
Tryptase Activity				
o-Phenoxybenzoyl-Pro-4-AmPhGly^{P}(OPh)$_2$	Naive (12 h)	210	0.55	20
	Control (12 h)	210	1.07	62
	Exposed (12 h)	210	2.04	73
	Naive (24 h)	210	0.53	24
	Control (24 h)	210	0.72	25
	Exposed (24 h)	210	11.3	92
Cathepsin B Activity				
Z-Leu-Abu-CONH(CH$_2$)$_3$-4-Mpl	Naive (3 h)	110	235	100
	Control (3 h)	110	264	100
	Exposed (3 h)	110	297	100
	Naive (6 h)		254	100
	Control (6 h)	110	230	100
	Exposed (6 h)	110	345	100
Calpain Activity				
Z-Leu-Abu-CONH(CH$_2$)$_3$-4-Mpl	Naive (24 h)	110	-1.08	-
	Control (24 h)	110	-0.33	-
	Exposed (24 h)	110	3.6	60

The inhibition of enzymatic activity of four proteases are summarized in Table III. The phosphonate Boc-Val-Pro-Val^{P}(OPh)$_2$

inhibited the elastase activity of both control and exposed samples equally well (86-92%). The phosphonate *o*-phenoxybenzoyl-Pro-4-AmPhGly^{P}(OPh)$_2$, a potent thrombin inhibitor inhibited the tryptase activity of exposed samples more potently than control samples (73-92 vs 25-62%). The α-ketoamide transition-state inhibitor Z-Leu-Abu-CONH-$(CH_2)_3$-4-Mpl completely abolished the cathepsin B activity of the control and exposed samples. This compound also inhibited calpain activity of exposed sample ca. 60%.

2. Effect of Pretreatment with Anti-inflammatory Drugs on the Protease Activity in Homogenates from the Mouse Ear Skin Model.

Samples from animals pretreated with three anti-inflammatory drugs (olvanil, retro-olvanil and indomethacin) prior to SM exposure had some reduced protease activities at various harvest times (12, 24 and 48 h) when compared with samples pretreated with ethanol (positive controls). Clearly, anti-inflammatory drugs have some effects on the proteolytic enzyme activity in mouse ear vesicant model

Skin tissues from the mouse ear were pretreated with ethanol (positive control), olvanil (structure in Fig. 12), retro-olvanil, or indomethacin (structure in Fig. 12) for 15 min prior to exposure to SM. Samples were harvested at various times and then the homogenates were assayed for serine and cysteine protease activities. The SM exposed tissue samples were obtained from animals (n = 10) at 6, 12, 24, 48 and 72 h postexposure from the right ear, while control samples pretreated with dichloromethane were obtained from the left ear. The samples of naive control (left and right ear) were obtained from animals which received no SM treatment (n = 10). There were 96 samples obtained at 48 h postexposure (including naive controls). Two animals died during the experiment. There are 80 samples harvested at 6, 12 and 72 h post SM exposure (no naive controls), and 100 samples were harvested at 24 h postexposure (including naive controls).

The enzyme activities of these homogenates were assayed with chromogenic thioester and fluorogenic AMC substrates. The results were shown in Table IV (at 6 h postexposure) and V (at 48 h postexposure) as well as Table 3 (at 12 h postexposure), 4 (at 24 h postexposure), and 5 (at 72 h postexposure) in the Appendices. The elastase activities of the homogenates are much higher when Boc-Ala-Ala-Ala-SBzl rather than MeO-Suc-Ala-Ala-Pro-Val-SBzl is used as the substrate. For chymase activity, the hydrolytic activities of Suc-Phe-Leu-Phe-SBzl in these homogenates are also higher than the activities of Suc-Ala-Ala-Pro-Phe-SBzl. Differences in enzymatic activity with different substrates may indicate that different or multiple elastases or chymases are being expressed in these animal models. Generally, enzyme activities of elastase (Fig. 13), tryptase (Fig. 14), chymase (Fig. 15), and calpain (Fig. 16) of exposed samples pretreated with ethanol are lower at 6, 12, 24 and 72 h postexposure than those at 48 h postexposure, while cathepsin B (Fig. 17) and

cathepsin H (Fig. 18) activities are similar at 6 and 48 h postexposure.

Elastase activities of exposed samples (pretreated with ethanol) are increased 4-93 times when compared to those of control samples at various harvest time (6-72 h). Tryptase, chymase and calpain activities of exposed samples are also gradually increased when the samples are harvested at longer postexposure times (48 and 72 h). At 48 h postexposure, the activities of elastase, tryptase, chymase, and calpain increased 3-85 times for the samples pretreated with ethanol when compared to naive control, while the cathepsin B and cathepsin H activities did not change significantly between the ethanol pretreated samples and naive control. This increase in proteolytic activity is probably indicative of a systemic inflammation in the samples of positive controls.

Various protease activities in exposed samples at 72 h postexposure decreased when compared to those at 48 h postexposure (Fig. 13-18). We discovered that samples obtained at 72 h postexposure lost some protease activities during a long period of storage in the freezer (-20 or -70 °C). Several exposed samples at 48 h postexposure were tested before and after 3 months storage at -20 °C, and the results indicated that elastase, chymase, calpain and cathepsin H lost 25-83% activity, while tryptase activity increased and cathepsin B activity did not change much (Table 6 in the Appendices).

Animals pretreated with several anti-inflammatory drugs had reduced levels of proteolytic enzymes compared to controls (Table V). At 48 h postexposure, animals pretreated with olvanil or indomethacin had lower elastase (45%; 29%), tryptase (18%; 6%), calpain (28%; 31%) and chymase (60-62%; 34-41%) activities than those pretreated with ethanol (100%). Samples pretreated with retro-olvanil had lower tryptase activity (52%) when compared to those pretreated with ethanol (100%). The olvanil and retro-olvanil pretreatment did not change the enzyme activities of cathepsin B and cathepsin H in the exposed samples, while exposed samples pretreated with indomethacin had lower cathepsin B and H activities. These three drugs also had some effects on various protease activities at different harvest times (6, 12, or 24 h). For example, at 6 h postexposure, only slight changes in enzyme activities were observed in samples after being pretreated with ethanol, olvanil, or indomethacin. Samples pretreated with retro-olvanil and harvested at 6 or 12 h postexposure had lower elastase, tryptase, and calpain activities. At 24 h postexposure, skin homogenates from animals which were pretreated with indomethacin in ethanol before SM exposure had lower elastase, tryptase, and calpain activities than those pretreated only with ethanol.

Table IV. Enzymatic Activities of Pretreated Mouse Ear Skin Homogenates at 6 h postexposure.[a]

Enzyme (Substrate)	Enzyme Activity (mOD/min or Fl/min)	
Pretreatment	Exposed Samples	Control Samples
Elastase (Boc-Ala-Ala-Ala-SBzl)		
ethanol	4.26 ± 0.69	1.05 ± 0.21
olvanil	14.70 ± 5.02	0.80 ± 0.34
indomethacin	4.70 ± 1.44	0.74 ± 0.27
retro-olvanil	1.83 ± 0.49	0.50 ± 0.12
(MeO-Suc-Ala-Ala-Pro-Val-SBzl)		
ethanol	0.01 ± 0.02	0.01 ± 0.02
olvanil	0.08 ± 0.03	0.06 ± 0.01
indomethacin	0.04 ± 0.01	0.07 ± 0.02
Tryptase		
ethanol	2.58 ± 0.26	2.33 ± 0.36
olvanil	3.59 ± 0.37	2.11 ± 0.08
indomethacin	2.39 ± 0.18	2.02 ± 0.14
retro-olvanil	0.86 ± 0.08	0.97 ± 0.36
Chymase (Suc-Phe-Leu-Phe-SBzl)		
ethanol	22.2 ± 3.09	17.6 ± 2.30
olvanil	24.0 ± 2.08	23.6 ± 1.43
indomethacin	16.7 ± 1.51	20.0 ± 1.77
retro-olvanil	19.0 ± 2.22	17.6 ± 1.84
(Suc-Ala-Ala-Pro-Phe-SBzl)		
ethanol	1.27 ± 0.15	1.38 ±0.11
olvanil	2.10 ± 0.35	1.07 ± 0.04
indomethacin	1.25 ± 0.16	1.11 ± 0.09
Calpain		
ethanol	2.02 ± 0.15	1.09 ± 0.18
olvanil	2.24 ± 1.25	0.59 ± 0.10
indomethacin	1.31 ± 0.19	0.83 ± 0.25
retro-olvanil	0.78 ± 0.18	0.94 ± 0.23
Cathepsin B		
ethanol	237 ± 22.1	206 ± 14.3
olvanil	214 ± 11.4	166 ± 9.7
indomethacin	198 ± 13.7	173 ± 16.9

retro-olvanil	207 ± 17.6	186 ± 11.6
Cathepsin H		
ethanol	198 ± 19.2	174 ± 19.3
olvanil	252 ± 16.3	207 ± 11.5
indomethacin	194 ± 14.0	199 ± 21.5
retro-olvanil	106 ± 23.6	116 ± 22.2

Table V. Enzymatic Activities of Pretreated Mouse Ear Skin Homogenates at 48 h postexposure.[a]

Enzyme (Substrate) In Pretreatment	Enzyme Activity (mOD/min or Fl/min) Exposed Samples	Control Samples	Naive Control
Elastase (Boc-Ala-Ala-Ala-SBzl)			
Ethanol	120 ± 19.2	1.29 ± 0.24	1.42 ± 0.28
olvanil	54.4 ± 8.23	1.66 ± 0.21	
indomethacin	35.2 ± 12.4	-0.37 ± 0.15	
retro-olvanil	116 ± 26.8	1.29 ± 0.23	
(MeO-Suc-Ala-Ala-Pro-Val-SBzl)			
ethanol	0.15 ± 0.04	0.08 ± 0.02	0.18 ± 0.02
olvanil	0.15 ± 0.03	0.10 ± 0.03	
indomethacin	0.08 ± 0.01	0.18 ± 0.03	
retro-olvanil	0.30 ± 0.07	0.09 ± 0.02	
Tryptase			
ethanol	6.45 ± 1.55	-0.47 ± 0.11	0.46 ± 0.26
olvanil	1.15 ± 0.26	0.29 ± 0.14	
indomethacin	0.40 ± 0.63	-1.10 ± 0.09	
retro-olvanil	3.38 ± 1.32	-1.9 ± 0.17	
Chymase (Suc-Phe-Leu-Phe-SBzl)			
ethanol	59.0 ± 7.2	20.0 ± 2.22	22.9 ± 1.92
olvanil	36.7 ± 4.3	30.1 ± 7.83	
indomethacin	20.3 ± 4.3	17.7 ± 2.61	
retro-olvanil	51.3 ± 11.6	17.7 ± 1.51	
(Suc-Ala-Ala-Pro-Phe-SBzl)			
ethanol	8.64 ± 1.05	0.96 ± 0.06	1.44 ± 0.24
olvanil	5.21 ± 1.06	1.56 ± 0.52	
indomethacin	3.56 ± 1.28	0.75 ± 0.09	
retro-olvanil	9.25 ± 1.26	0.71 ± 0.08	
Calpain			
ethanol	25.1 ± 4.81	1.03 ± 0.16	1.76 ± 0.16
olvanil	7.0 ± 1.38	3.66 ± 1.48	
indomethacin	7.8 ± 2.57	0.77 ± 0.17	
retro-olvanil	22.2 ± 6.15	0.95 ± 0.20	
Cathepsin B			
ethanol	215 ± 26.5	200 ± 15.1	262 ± 21.0
olvanil	188 ± 17.8	233 ± 32.0	
indomethacin	159 ± 21.2	211 ± 12.1	
retro-olvanil	251 ± 36.7	202 ± 17.5	

Cathepsin H			
ethanol	207 ± 33.9	167 ± 18.7	152 ± 11.8
olvanil	190 ± 20.7	180 ± 36.1	
indomethacin	110 ± 16.9	19.7	
retro-olvanil	224 ± 30.4	100 ± 9.76	

3. Effect of Protease Inhibitors on the Protease Activity of the Pretreated Mouse Ear Skin Homogenates.

The exposed samples pretreated with protease inhibitors (3-chloroisocoumarin, Suc-Val-Pro-Phe^{P}(OPh)$_2$ and a combination of 3-chloroisocoumarin and olvanil) had reduced protease activities at various harvest time (12, 24, 48 or 72 h) when compared to the samples pretreated with ethanol (positive controls). Clearly, protease inhibitors affect the proteolytic enzyme activity in mouse ear vesicant model.

Skin tissues from mouse ear exposed to SM were pretreated with ethanol (positive control), 3-chloroisocoumarin, Suc-Val-Pro-Phe^{P}(OPh)$_2$ (structures in Fig. 12) and a combination of 3-chloroisocoumarin and olvanil, for 15 min, and then the homogenates were assayed for serine and cysteine protease activities after harvesting tissue. The tissue samples were obtained from animals (n = 10) at 6, 12, 24, 48 and 72 h postexposure from the right ear (SM exposed), while control samples were obtained from the left ear (pretreated with dichloromethane). The samples of naive control (left and right ear) were obtained from animals which received no SM treatment (n = 10). There were 100 samples obtained at 6, and 48 h postexposure (including naive controls). There were 80 samples harvested at 12, 24, and 72 h post SM exposure (no naive controls).

The enzyme activities of these homogenates were assayed with the chromogenic thioester and fluorogenic AMC substrates. The results were shown in Table VI (at 6 h postexposure) and VII (at 72 h postexposure) as well as Table 7 (at 12 h postexposure), 8 (at 24 h postexposure), and 9 (at 48 h postexposure) in the Appendices. Generally, activities of elastase (Fig. 19), tryptase (Fig. 20), chymase (Fig. 21), calpain (Fig. 22) and cathepsin H (Fig. 23) in the exposed samples pretreated with ethanol are lower at 6, 12, 24 and 48 h postexposure than those at 72 h postexposure, while there is no trend in cathepsin B activity (Fig. 24).

Elastase activities of exposed samples (pretreated with ethanol) were increased 15-70 times when compared to those of control samples at various harvest time (6-72 h). Tryptase, and chymase activities of exposed samples are also gradually increased when the samples are harvested at longer postexposure time (48 and 72 h). At 72 h postexposure, the activities of elastase,

tryptase, chymase, calpain and cathepsin H were the highest for the samples pretreated with ethanol, while the cathepsin B activities did not vary much between the ethanol pretreated samples and naive control. This increase in proteolytic activity probably indicates a systemic inflammation in the samples of positive control.

Pretreatment of animals with 3-chloroisocoumarin or (3-chloroisocoumarin + olvanil) did not have much effect on exposed samples harvested at 6 h (Table VI) and 12 h postexposure (Table 7), but the samples had reduced levels of proteolytic enzyme activities when compared to positive controls at 24 h (Table 8), 48 h (Table 9), and 72 h postexposure (Table VII). At 12 h postexposure, skin homogenates which were pretreated with phosphonate in ethanol before SM exposure had lower chymase activity than those pretreated only with ethanol. At 24 h postexposure, samples pretreated with 3-chloroisocoumarin, phosphonate, or (3-chloroisocoumarin + olvanil) had lower elastase activity when compared to samples only pretreated with ethanol; Samples pretreated with phosphonate, or (3-chloroisocoumarin + olvanil) had lower chymase activity when compared to samples only pretreated with ethanol. At 48 h postexposure, samples pretreated with phosphonate had lower elastase, chymase, cathepsin B and cathepsin H activities when compared to samples only pretreated with ethanol; Samples pretreated with 3-chloroisocoumarin had lower elastase activity when compared to samples only pretreated with ethanol. At 72 h postexposure, animals pretreated with 3-chloroisocoumarin or (3-chloroisocoumarin + olvanil) had lower elastase (41%; 36%), tryptase (35%; 35%), calpain (44%; 41%) and chymase (68%; 40%) activities than those pretreated with ethanol (100%). Samples pretreated with Suc-Val-Pro-Phe^{P}(OPh)$_2$ had lower chymase (61%) and cathepsin B activity (29%) when compared to those pretreated with ethanol (100%).

Table VI. Enzymatic Activities of Pretreated Mouse Ear Skin Homogenates at 6 h postexposure.[a]

Enzyme (Substrate) In Pretreatment	Enzyme Activity (mOD/min or Fl/min) Exposed Samples	Control Samples	Naive Control
Elastase			
ethanol	0.66 ± 0.17	0.04 ± 0.03	0.25 ± 0.06
3-chloroisocoumarin	5.00 ± 1.7	0.02 ± 0.05	
Suc-Val-Pro-Phe^{P}(OPh)$_2$	0.87 ± 0.56	0.18 ± 0.05	
3-chloroisocoumarin + olvanil	3.62 ± 0.96	0.06 ± 0.05	
Tryptase			
ethanol	0.40 ± 0.06	0.38 ± 0.02	0.15 ± 0.07
3-chloroisocoumarin	1.02 ± 0.19	0.37 ± 0.04	
Suc-Val-Pro-Phe^{P}(OPh)$_2$	0.59 ± 0.16	0.40 ± 0.03	
3-chloroisocoumarin + olvanil	1.12 ± 0.24	0.30 ± 0.09	
Chymase			
ethanol	17.5 ±1.73	19.3 ± 1.59	20.1 ± 1.18
3-chloroisocoumarin	20.9 ± 2.29	19.9 ± 1.86	
Suc-Val-Pro-Phe^{P} (OPh)$_2$	14.3 ± 0.66	19.8 ± 1.80	
3-chloroisocoumarin + olvanil	17.8 ± 2.4	22.8 ± 1.60	
Calpain			
ethanol	-0.19 ± 0.18	-0.11 ± 0.13	0.93 ± 0.17
3-chloroisocoumarin	0.41 ± 0.15	-0.23 ± 0.11	
Suc-Val-Pro-Phe^{P}(OPh)$_2$	0.97 ± 0.20	0.06 ± 0.14	
3-chloroisocoumarin + olvanil	2.68 ± 0.30	1.14 ± 0.18	
Cathepsin B			
ethanol	219 ± 16.8	211 ± 16.6	246 ± 13
3-chloroisocoumarin	238 ± 21.4	201 ± 12.8	
Suc-Val-Pro-Phe^{P}(OPh)$_2$	265 ± 11.5	232 ± 11.9	
3-chloroisocoumarin + olvanil	312 ± 22.8	282 ± 21.9	
Cathepsin H			
ethanol	82.3 ± 7.8	92.7 ± 8.0	86.3 ± 5.92
3-chloroisocoumarin	120 ± 11.6	93.8 ± 7.9	
Suc-Val-Pro-Phe^{P}(OPh)$_2$	81 ± 7.78	107 ± 7.32	
3-chloroisocoumarin + olvanil	114 ± 14.3	94.7 ± 7.25	

Table VII. Enzymatic Activities of Pretreated Mouse Ear Skin Homogenates at 72 h postexposure.[a]

Enzyme (Substrate) In Pretreatment	Enzyme Activity (mOD/min or Fl/min) Exposed Samples	Control Samples
Elastase		
Ethanol	74.8 ± 16.5	1.07 ± 0.07
3-chloroisocoumarin	30.4 ± 5.66	1.09 ± 0.06
Suc-Val-Pro-Phe^{P}(OPh)$_2$	61.7 ± 13.4	1.07 ± 0.13
3-chloroisocoumarin + olvanil	26.9 ± 4.16	0.71 ± 0.20
Tryptase		
ethanol	8.0 ± 1.28	0.78 ± 0.10
3-chloroisocoumarin	2.77 ± 0.35	0.57 ± 0.07
Suc-Val-Pro-Phe^{P}(OPh)$_2$	11.0 ± 1.32	1.78 ± 0.68
3-chloroisocoumarin + olvanil	2.80 ± 0.43	0.62 ± 0.07
Chymase (Suc-Phe-Leu-Phe-SBzl)		
ethanol	64.6 ± 11.0	14.4 ± 1.16
3-chloroisocoumarin	43.7 ± 7.06	18.0 ± 1.69
Suc-Val-Pro-Phe^{P}(OPh)$_2$	39.2 ± 7.66	18.6 ± 2.08
3-chloroisocoumarin + olvanil	25.8 ± 3.40	14.4 ± 1.61
Calpain		
ethanol	6.45 ± 1.11	-0.18 ± 0.15
3-chloroisocoumarin	2.83 ± 0.47	0.14 ± 0.15
Suc-Val-Pro-Phe^{P}(OPh)$_2$	5.68 ± 1.07	-0.07 ± 0.12
3-chloroisocoumarin + olvanil	2.66 ± 0.33	0.03 ± 0.16
Cathepsin B		
ethanol	300 ± 76.6	163 ± 11.8
3-chloroisocoumarin	353 ± 62.3	199 ± 17.4
Suc-Val-Pro-Phe^{P}(OPh)$_2$	87.2 ± 27.2	166 ± 13.9
3-chloroisocoumarin + olvanil	273 ± 38.9	107 ± 9.3
Cathepsin H		
ethanol	206 ± 6.46	84.1 ± 6.46
3-chloroisocoumarin	216 ± 35.4	101 ± 11.0
Suc-Val-Pro-Phe^{P}(OPh)$_2$	151 ± 30.8	121 ± 13.2
3-chloroisocoumarin + olvanil	96.8 ± 25.9	73.7 ± 9.65

II. Euthymic Hairless Mouse Model.

Skin homogenates from euthymic hairless mice after exposure to SM were assayed for several protease activities using sensitive chromogenic and fluorogenic substrates. We have shown that several protease activities of exposed homogenates are increased due to SM exposure at various harvest time. The inhibitory effects of three synthetic inhibitors on the proteolytic activity of the homogenates were also found in this model.

Tissue homogenates prepared from either sham-treated or SM exposed skin of euthymic hairless mice were assayed with serine and cysteine protease activities (Table VIII). The tissue samples were obtained from animals (n = 8) at 2, 6, and 24 h postexposure. Each animal had four exposure sites: two sites were controls and two sites were SM-exposed. There were a total of 96 samples, each value is the average of 16 data points from 16 samples at two sites on 8 different animals.

At 6 h postexposure, exposed samples had higher thrombin (Fig. 25), calpain (Fig. 26) and cathepsin H (Fig. 27) activities when compared to the control samples. At 24 h postexposure, exposed samples had higher elastase (Fig. 28) and plasmin (Fig. 29) activities when compared to the control samples. There are no difference in tryptase (Fig. 30), chymase (Fig. 31) and cathepsin B (Fig. 32) activities between the exposed and control samples. The same three protease inhibitors were used to inhibit protease activity of the tissue homogenates and the results were shown in Table IX. The Val phosphonate completely inhibited the elastase activity of exposed and control samples, and the ketoamide also abolished the cathepsin B and calpain activities of exposed and control samples. However, the amidine phosphonate only partially inhibited the tryptase activity of the exposed and control samples.

Table VIII. Enzymatic Activities of Euthymic Hairless Mouse Homogenates.

Hours Postexposure	Enzyme Activity (mOD/min or Fl/min)	
	Exposed Samples	Control Samples
Elastase		
2	0.45 ± 0.15	0.64 ± 0.31
6	0.41 ± 0.06	0.80 ± 0.14
24	1.30 ± 0.15	0.59 ± 0.12
Tryptase		
2	1.03 ± 0.03	1.00 ± 0.27
6	1.05 ± 0.14	1.19 ± 0.24
24	0.96 ± 0.08	1.01 ± 0.06
Chymase		
2	28.3 ± 4.86	25.1 ± 2.96
6	23.7 ± 1.38	27.4 ± 4.45
24	30.9 ± 8.90	48.6 ± 13.8
Plasmin		
2	1.13 ± 0.27	-1.91 ± 1.57
6	-0.78 ± 1.51	-0.06 ± 0.58
24	2.62 ± 0.38	-2.40 ± 1.31
Thrombin		
2	12.8 ± 3.43	20.8 ± 5.12
6	40.9 ± 12.0	19.0 ± 2.64
24	12.8 ± 2.0	12.4 ± 2.94
Calpain		
2	0.38 ± 0.33	-0.23 ± 0.18
6	1.56 ± 0.22	0.56 ± 0.04
24	0.37 ± 0.48	0.01 ± 0.84
Cathepsin B		
2	784 ± 48.1	794 ± 25.8
6	927 ± 61.1	917 ± 180
24	1090 ± 135	1178 ± 119
Cathepsin H		
2	24.0 ± 1.72	21.2 ± 1.55
6	67.0 ± 6.89	39.2 ± 5.12
24	25.7 ± 7.78	30.0 ± 7.28

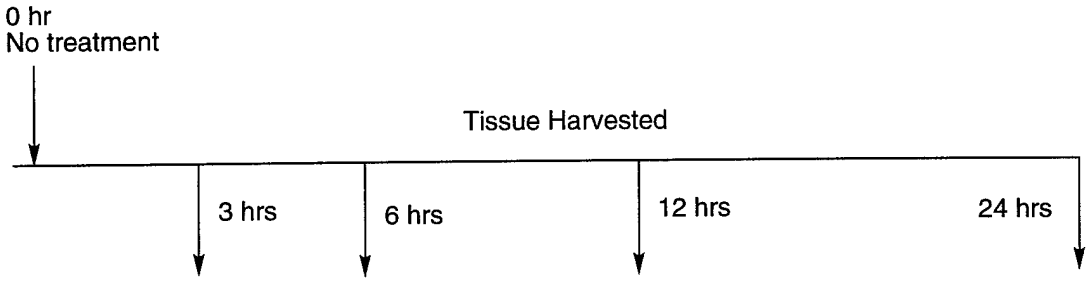
Table IX. Inhibition of Proteolytic Enzyme Activities in Homogenates of Euthymic Hairless Mouse Exposed to SM.

Inhibitors	Samples	Inh. Con. (μM)	Enz. Act. (-I)	% Inh.
Elastase Activity				
Boc-Val-Pro-Val^{P}(OPh)$_2$	Control (24 h)	45	0.21	100
	Exposed (24 h)	45	1.05	100
Tryptase Activity				
o-Phenoxybenzoyl-Pro-4-AmPhGly^{P}(OPh)$_2$	Control (24 h)	227	0.71	67
	Exposed (24 h)	227	0.89	49
Thrombin Activity				
o-Phenoxybenzoyl-Pro-4-AmPhGly^{P}(OPh)$_2$	Control (24 h)	227	3.24	26
	Exposed (24 h)	227	4.99	50
Cathepsin B Activity				
Z-Leu-Abu-CONH(CH$_2$)$_3$-4-Mpl	Control (6 h)	114	686	100
	Exposed (6 h)	114	939	100
Calpain Activity				
Z-Leu-Abu-CONH(CH$_2$)$_3$-4-Mpl	Control (6 h)	114	2.25	95
	Exposed (6 h)	114	2.85	100

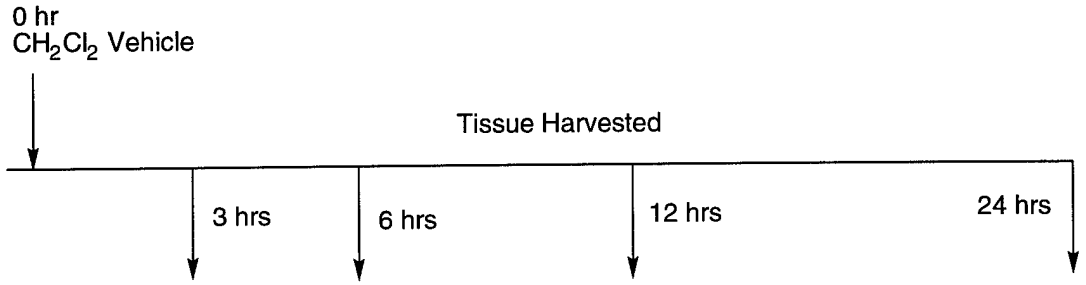
Graphical Summary of Research Results With Animal Models

Proteases in the Mouse Ear Vesicant Model

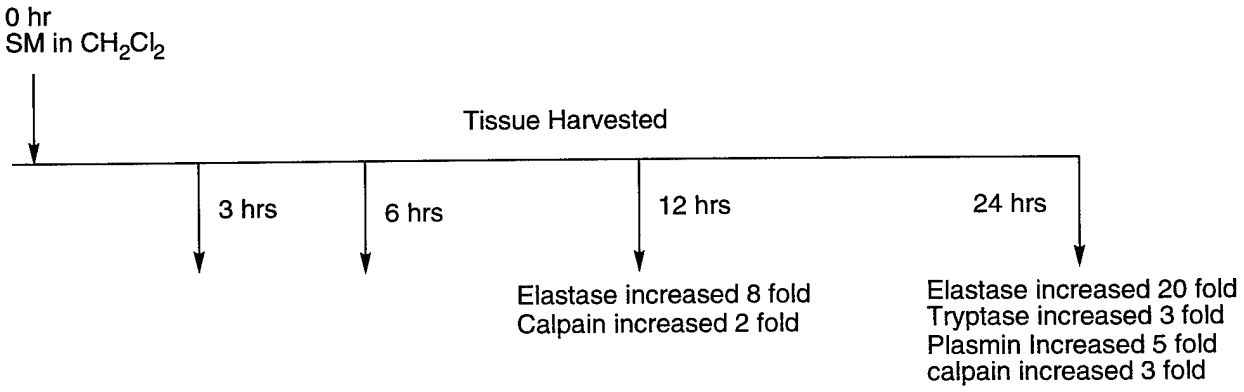
Naive Controls



Control (Left Ear)

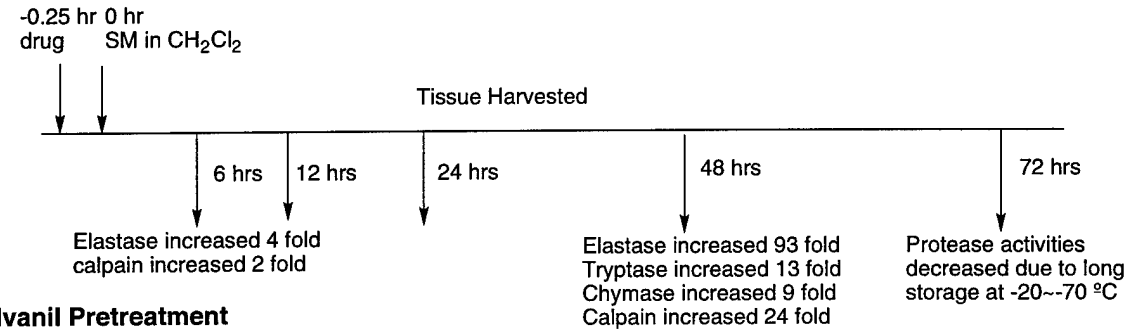


Exposed (Right Ear)

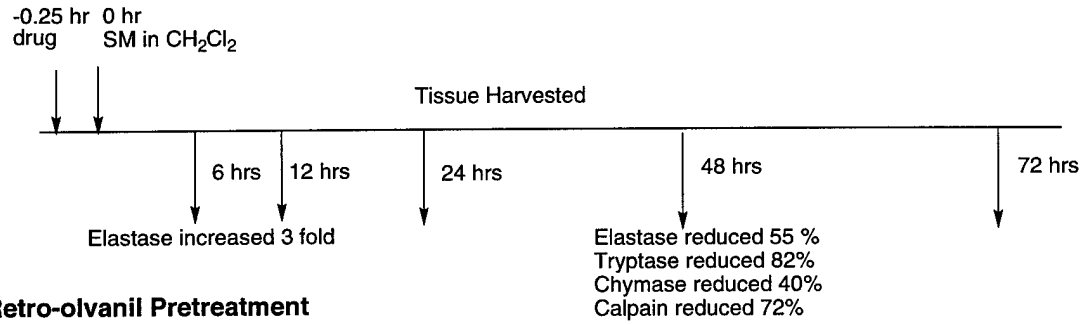


Mouse Ear Vesicant Model with Pretreatment

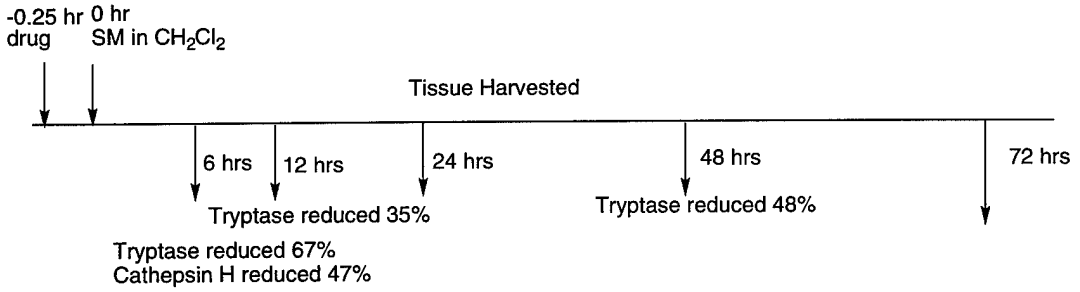
Ethanol Pretreatment



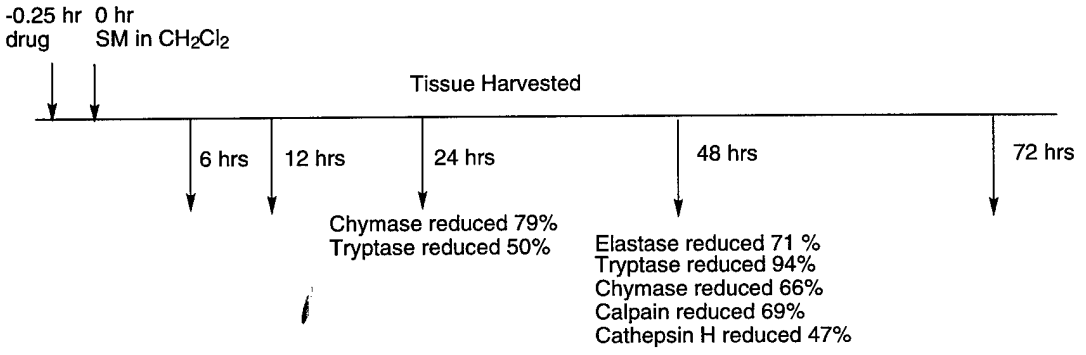
Olvanil Pretreatment



Retro-olvanil Pretreatment

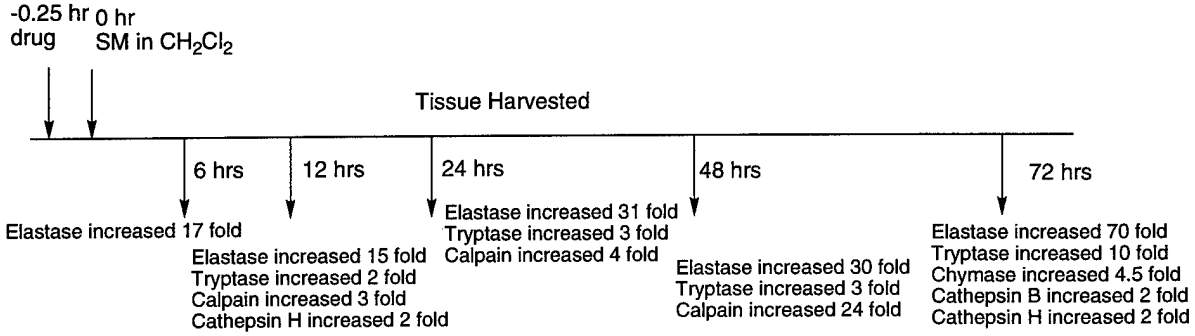


Indomethacin Pretreatment

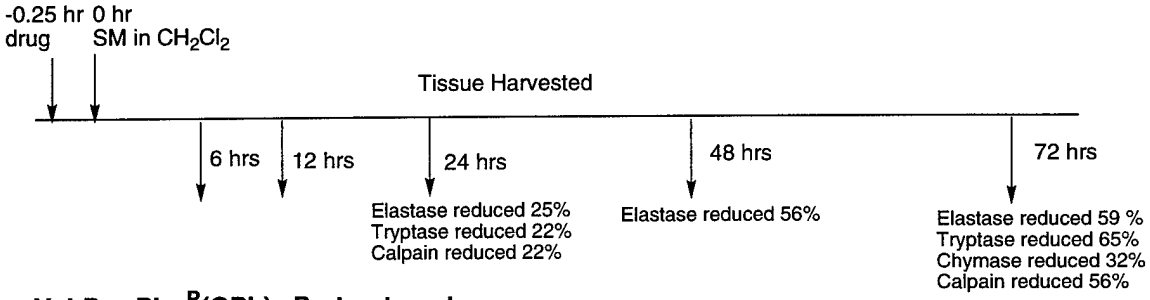


Mouse Ear Vesicant Model with Pretreatment

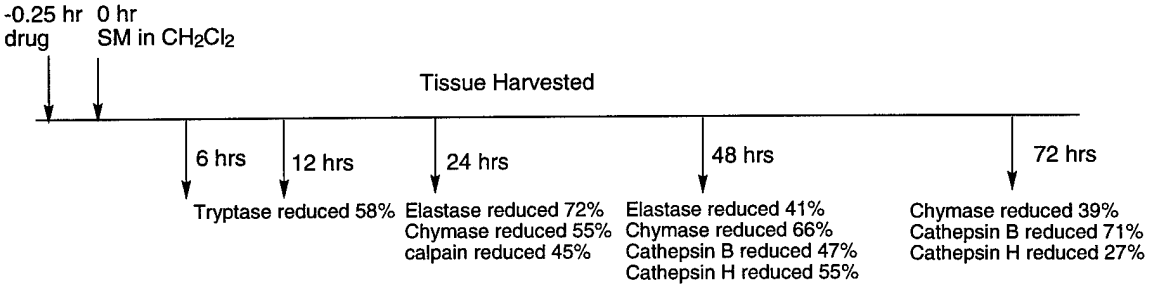
Ethanol Pretreatment



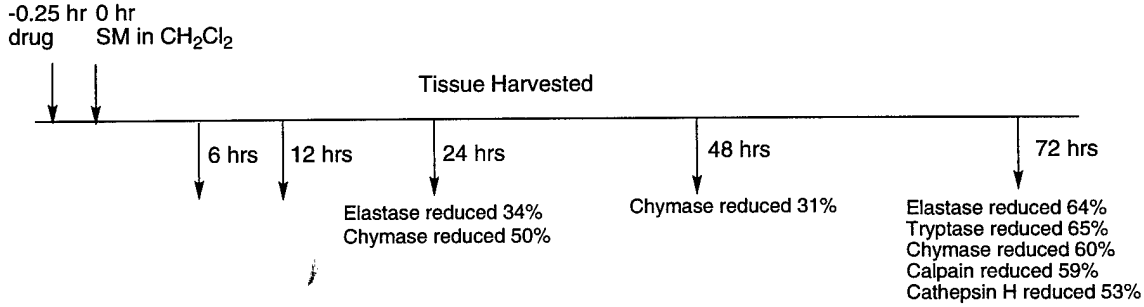
3-Chloroisocoumarin Pretreatment



Suc-Val-Pro-Phe^{P}(OPh)$_2$ Pretreatment

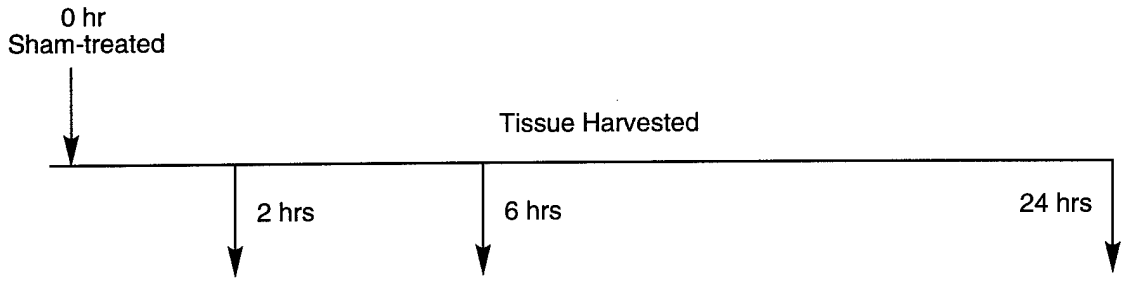


3-Chloroisocoumarin + Olvanil Pretreatment

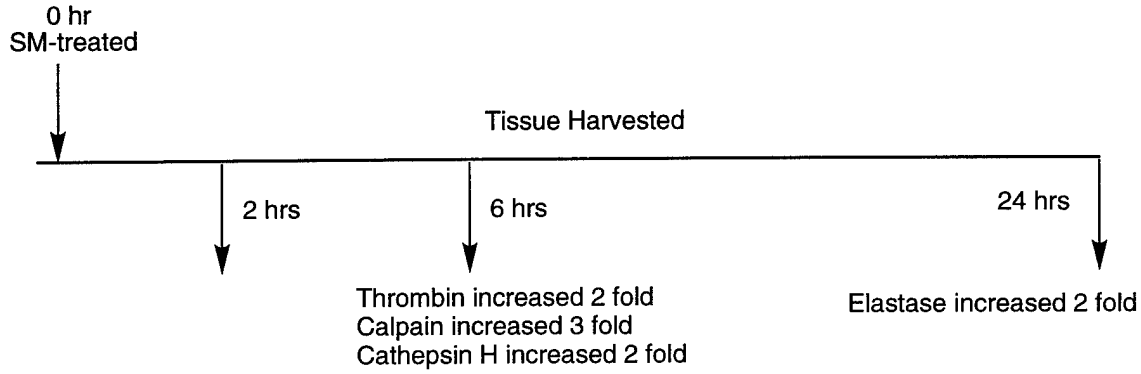


Proteases in the Euthymic Hairless Mouse Vesicant Model

Controls (2 sites on the dorsal surface)



Exposed (2 sites on the dorsal surface)



DISCUSSION

I. Mouse Ear Vesicant Model with No Pretreatment.

Protease Activities. Synthetic peptide substrates have been used previously for detecting protease activities in lymphocytes and homogenates of hairless guinea pig skin exposed to SM.[5,6,7] The SM exposed skin homogenate samples from the hairless guinea pig model showed enhanced elastase and tryptase activities, while the chymase and Asp-ase (cleaves after Asp) activities in these samples were not increased.[6]

In the mouse ear vesicant model, evaluation of endpoint responses (e.g. edema, histopathological responses) at various SM doses and time points indicated that a dose of 0.16 mg/ear SM and 24 h postexposure would be optimal conditions for future testing.[11] We found that the homogenates obtained at 0.16 mg/ear SM exposure and 24 h postexposure had higher elastase, tryptase and calpain activities when compared to the control samples. Enhancement of elastase and tryptase activities was also found in previous experiments with homogenates of skin from SM exposed hairless guinea pig model[6] and mouse ear model. The increase in elastase activity in the exposed samples indicates a general inflammatory response in the skin area exposed to SM. There was no clear trend for cathepsin B, cathepsin H, and chymase activities between control and exposed samples. All homogenates contained high cathepsin B and cathepsin H activities and some chymase activity.

Metalloprotease activities such as PUMP, collagenase, and stromelysin were measured at pH 7.5 and 37 °C using a Mca peptide substrate, while gelatinase activity was measured at pH 6.0 using the same substrate.[20] We found that the exposed samples at 24 h postexposure had higher gelatinase activities than control samples; however, other metalloprotease activities were not detected at pH 7.5. Assay conditions using more homogenate volume, longer assay time, and higher temperature are needed to detect other metalloprotease activities in the exposed samples.

Inhibition of Protease Activities. Three different protease inhibitors blocked SM induced proteolytic activity. The phosphonate Boc-Val-Pro-Val^{P}(OPh)$_2$ is a potent inhibitor of elastase, it effectively blocked the elastase activity of the homogenates. However, *o*-phenoxybenzoyl-Pro-4-AmPhGly^{P}(OPh)$_2$, a potent thrombin inhibitor, only inhibited the tryptase activity of exposed samples effectively and not the tryptase activity of controls. This may indicate that a unique tryptase is expressed upon exposure to SM. The α-ketoamide Z-Leu-Abu-CONH-(CH$_2$)$_3$-4-mpl completely abolished the cathepsin B activity of the homogenates. However, this compound inhibited the calpain activity less effectively. These synthetic inhibitors substantially reduced the proteolytic activities which are elevated when skin is exposed to SM.

II. Mouse Ear Vesicant Model with Antiiflammatory Drug Pretreatment.

Several anti-inflammatory drugs such as olvanil, retro-olvanil, and indomethacin were used to treat the skin of the mouse ear prior to the SM exposure. Olvanil and retro-olvanil belonged to a class of compounds called vanilloids which are capsaicin analogs, the pungent ingredient of hot peppers, which were developed as a novel class of analgesics. Capsaicin analogs are known to inhibit neurogenic inflammation by blocking neurokinin release. Indomethacin is a non-steroidal drug with anti-inflammatory, antipyretic and analgesic properties. The anti-inflammatory activity of indomethacin is due to its inhibition of the cyclooxygenase activity of prostagladin H synthetase which catalyzes the first two steps in prostaglandin synthesis. These three drugs have previously been shown to have some effect in decreasing the inflammatory response in the mouse ear vesicant model. Therefore, they were used to study their effects on various proteolytic enzyme activities in the skin homogenates in order to correlate the *in vitro* effect on proteases with their *in vivo* effect.

Protease activity measurements at 48 h postexposure show that the elastase, tryptase, chymase, and calpain activities of samples pretreated with ethanol increased 3-28 times when compared to the activities at 6 h postexposure, while cathepsin B and cathepsin H activities did not vary much between those two time points. Several addition time points (12, and 24 h) in this series of animals were also analyzed. Protease activities in the exposed samples are gradually increased when the harvest time of postexposure is increased. These results indicate that SM exposure might have a latent effect on the protease activities. Various protease activities of the exposed samples at 72 h postexposure were smaller than those at 48 h postexposure. This is due to the fact that samples obtained at 72 h lost protease activity during long period of storage in the freezer.

Olvanil and indomethacin treatment were effective in reducing elastase, tryptase, chymase and calpain activities by 40-90% in the exposed samples at 48 h postexposure. Retro-olvanil was only effective in reducing tryptase activity by 50% but was not effective with other proteolytic activities. Indomethacin decreased the cathepsin B and cathepsin H activities by 26 and 47%, respectively. These results indicate that the three antiinflammatory drugs studied have different mechanism of action due to their differential effects on protease activities.

Variation in the hydrolytic activities between Boc-Ala-Ala-Ala-SBzl and MeO-Suc-Ala-Ala-Pro-Val-SBzl which measures elastase activity was detected with the former being more reactive than the latter. This may indicate that the specificity of mouse elastase is different than human elastase which prefers Val to Ala at the P1 site. This may also indicate mouse elastase does not like Pro at the P2 site. The chymase activity of mouse homogenates was detected using either Suc-Phe-Leu-Phe-SBzl or Suc-Ala-Ala-Pro-Phe-

SBzl with the former being more reactive. This result also indicates that mouse chymase prefers Leu to Pro at the P2 site.

III. Mouse Ear Vesicant Model with Protease Inhibitor Pretreatment.

Two protease inhibitors, 3-chloroisocoumarin and Suc-Val-Pro-Val^{P}(OPh)$_2$, from the PI's laboratory and a combination of 3-chloroisocoumarin and olvanil were used for pretreatment of the skin prior to SM exposure in the mouse ear model. 3-Chloroisocoumarin is a general serine protease inhibitor,[21] and Suc-Val-Pro-Val^{P}(OPh)$_2$ is a specific inhibitor for chymase,[15] thus they are expected to inhibit only some of the protease activities in the exposed samples. Combination drug treatment was also examined.

Protease activity measurements at 72 h postexposure show that the elastase, tryptase, and chymase activities of samples pretreated with ethanol increased 4-113 times when compared to the activities at 6 h postexposure, while cathepsin B and cathepsin H activities did not vary much between those two time points. Several additional time points (12, 24, and 48 h) in this series of animals were also analyzed. Protease activities such as elastase, tryptase and chymase activities in the exposed samples were gradually increased when the harvest time postexposure increased from 6 h to 72 h. These results indicate that SM exposure might have a latent effect on the protease activities.

All three drugs did not have much effect on various protease activities in the early postexposure stages of toxicity (6 and 12 h). However, they reduced protease activities in exposed samples after the samples were obtained at longer postexposure times (24-72 h). These results indicate that the inhibitors were more effective in the later stages of toxicity following SM exposure. Since 3-chloroisocoumarin is a general protease inhibitor, it is expected that this compound reduced elastase, tryptase and chymase activities of exposed samples at various postexposure time. The phosphonate is specific chymase inhibitor, it is also expected that this compound reduced the chymase activity of exposed samples. However, it is surprising that the phosphonate also had some effects on cathepsin B and cathepsin H activities in the exposed samples since the phosphonate is known to inhibit only serine proteases but not the cysteine proteases. The combination of 3-chloroisocoumarin and olvanil pretreatment also reduced several protease activities in the exposed samples harvested at longer postexposure times (72 h), but the effect is not greater than using either drug alone.

IV. Comparison Of Protease Activities in the Euthymic Hairless Mouse Model And the Mouse Ear Vesicant Model.

Like the mouse ear vesicant model, exposed samples from the euthymic hairless mouse model have higher elastase and calpain

activities when compared to the control samples. Nearly identical chymase and cathepsin B activities were found in both samples. The three protease inhibitors tested also have a similar inhibitory effect samples from both models; however, there is a difference in the enhancement of tryptase activity which was observed in samples from the SM exposed mouse ear model but not in samples from the euthymic hairless mice. In the euthymic hairless mice several protease activities such as cathepsin H, thrombin, and calpain from exposed samples were increased most at 6 h postexposure compared to 24 h in the mouse ear model.

CONCLUSION

The serine proteases, elastase, chymase, and tryptase, and the cysteine proteases, cathepsin B, cathepsin H, and calpain as well as metalloprotease activities of skin homogenates from mouse ear exposed to SM were assayed with synthetic chromogenic or fluorogenic substrates. All the SM exposed samples harvested at longer postexposure time such as 24 h had higher activities of elastase, tryptase and calpain than the control samples. The increase in protease activities in the exposed samples indicates a general inflammatory response in skin area exposed to sulfur mustard. Both unexposed and SM exposed skin homogenates have distinct cathepsin B and cathepsin H enzyme activities and some chymase activity.

Enhancement of elastase and calpain activities in the skin homogenates from the euthymic hairless mice exposed to SM was also found in samples from the mouse ear vesicant model, although several other protease activities from exposed samples are also increased at different postexposure times in the mouse ear vesicant model.

Three different inhibitors were used to block the proteolytic activity of the skin homogenates from both models. The phosphonate effectively abolished elastase activities in the exposed samples, and the amidine phosphonate inhibited some of the tryptase activities in these samples. A cysteine protease inhibitor completely inhibited cathepsin B and some calpain activities of exposed samples. It is likely that protease inhibitors and anti-inflammatory drugs singly or in combination will be therapeutically useful for reducing tissue injury resulting from SM exposure.

Pretreatment of mouse ear skin with anti-inflammatory drugs, protease inhibitors, or a combination of both prior to the SM exposure decreased several proteolytic enzyme activities of the skin homogenates. This indicates there is a correlation between the *in vitro* activity of these drugs and their *in vivo* activity. The enhancement of protease activities in the skin homogenates with ethanol pretreatment at 48 or 72 h postexposure when compared to those at 6 h postexposure also indicates that a latent effect occurs after SM exposure.

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ABBREVIATIONS

[a]Abbreviations: AMC, 7-amino-4-methylcoumarin; (4-AmPhGly)^{P}(OPh)$_2$, diphenyl [(4-amidinophenyl)glycyl)]phosphonate; Boc, *t*-butyloxycarbonyl; Brij 35, polyoxyethylene 23 lauryl ether; Dpa, N-3-(2,4-dinitrophenyl)-L-2,3-diaminopropionyl; DTNB, 5,5'-dithiobis(2-nitrobenzoic acid); DTT, dithiothreitol; EDTA, ethylenediaminetetraacetic acid; HEPES, N-(2-hydroxyethyl)piperazin-N'-(2-ethanesulfonic acid); Mca, (7-methoxycoumarin-4-yl)acetyl; MMP, matrix metalloproteases; Mpl, morpholinyl; PIPES, Piperazine-N,N'-bis(2-ethanesulfonic acid); PUMP, punctuated metalloprotease; SBzl, thiobenzyl; SM, sulfur mustard; Suc, succinyl; Tos, tolunesulfonyl; Z, benzyloxycarbonyl.

Publications

Cutaneous Protease Activity In the Mouse Ear Vesicant Model, Powers, J. C., Kam, C.-M., Ricketts, K. M., and Casillas, R. P. (1999) *J. Applied Toxicology*, accepted.

Personnel

James C. Powers, PI
Chih-Min Kam, Research Scientist
Karrie Adlington, Graduate Research Assistant

Figure Legends

Figure 1. Elastase activities of homogenates from mouse ear skin exposed to SM.

Figure 2. Elastase activities of homogenates from mouse ear skin exposed to SM using vaious amount of homogenate in the assays.

Figure 3. Tryptase activities of homogenates from mouse ear skin exposed to SM using Z-Arg-SBzl as the substrate.

Figure 4. Tryptase activities of homogenates from mouse ear skin exposed to SM using Z-Arg-AMC as the substrate.

Figure 5. Thrombin activities of homogenates from mouse ear skin exposed to SM.

Figure 6. Plasmin activities of homogenates from mouse ear skin exposed to SM.

Figure 7. Calpain activities of homogenates from mouse ear skin exposed to SM.

Figure 8. Cathepsin B activities of homogenates from mouse ear skin exposed to SM.

Figure 9. Cathepsin H activities of homogenates from mouse ear skin exposed to SM.

Figure 10. Chymase activities of homogenates from mouse ear skin exposed to SM.

Figure 11. Metalloproteinase activities of homogenates at pH 6.0 from mouse ear skin exposed to SM using vaious amount of homogenate in the assays.

Figure 12. Structures of synthetic protease inhibitors and antiinflammatory drugs.

Figure 13. Elastase activities of homogenates from pretreated mouse ear skin exposed to SM using Boc-Ala-Ala-Ala-SBzl as the substrate.

Figure 14. Tryptase activities of homogenates from pretreated mouse ear skin exposed to SM.

Figure 15. Chymase activities of homogenates from pretreated mouse ear skin exposed to SM using Suc-Phe-Leu-Phe-SBzl as the substrate.

Figure 16. Calpain activities of homogenates from pretreated mouse ear skin exposed to SM.

Figure 17. Cathepsin B activities of homogenates from pretreated mouse ear skin exposed to SM.

Figure 18. Cathepsin H activities of homogenates from pretreated mouse ear skin exposed to SM.

Figure 19. Elastase activities of homogenates from pretreated mouse ear skin exposed to SM using Boc-Ala-Ala-Ala-SBzl as

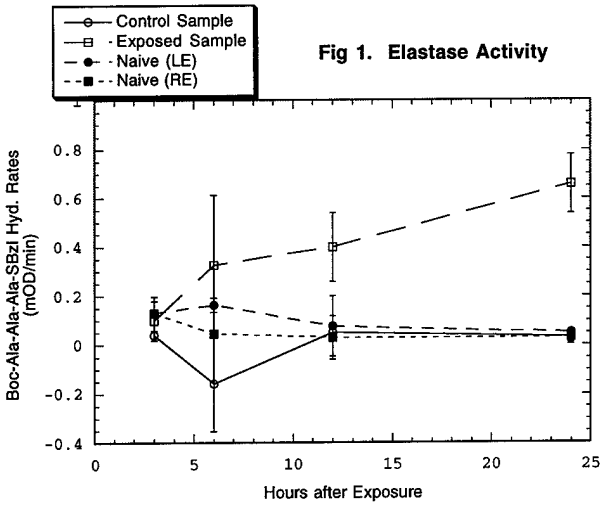


Fig 1. Elastase Activity

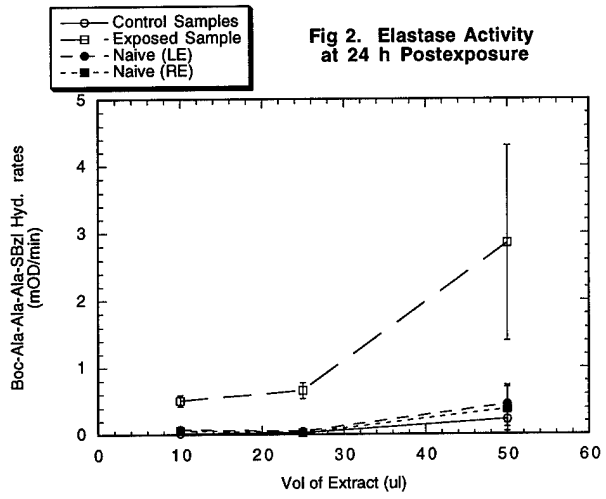


Fig 2. Elastase Activity at 24 h Postexposure

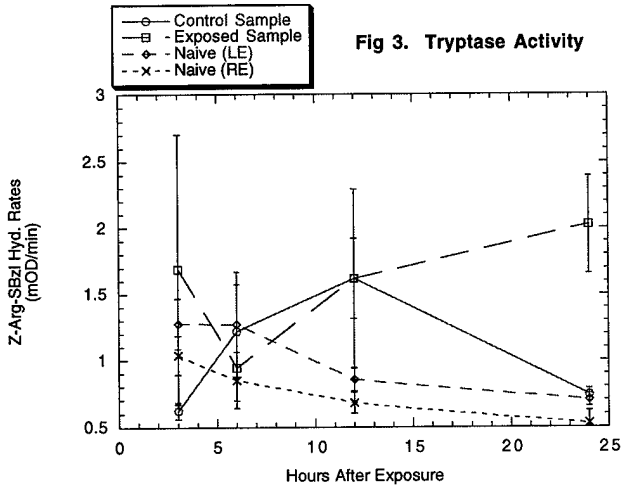


Fig 3. Tryptase Activity

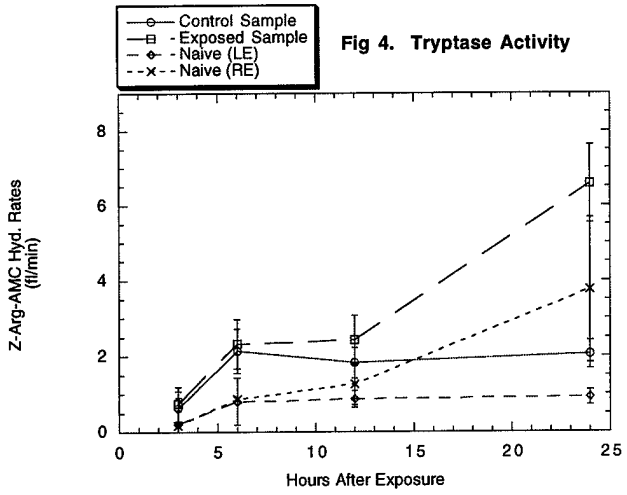


Fig 4. Tryptase Activity

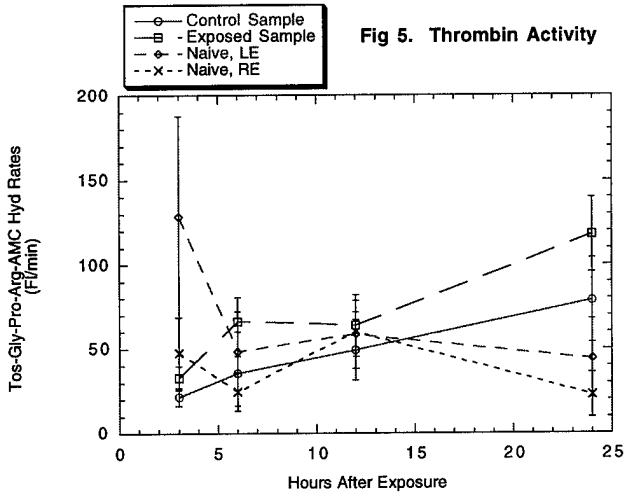


Fig 5. Thrombin Activity

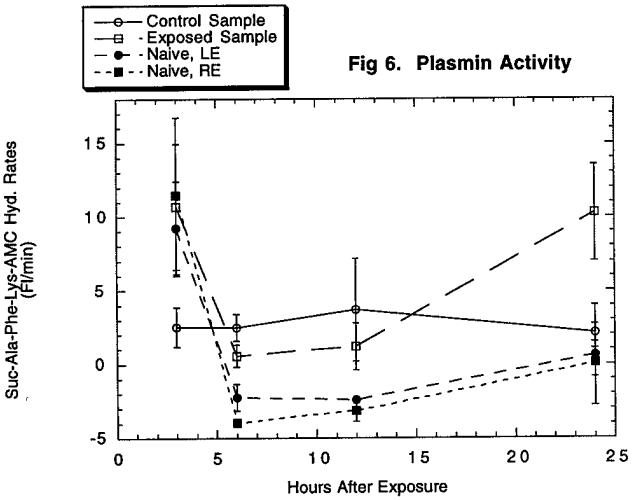


Fig 6. Plasmin Activity

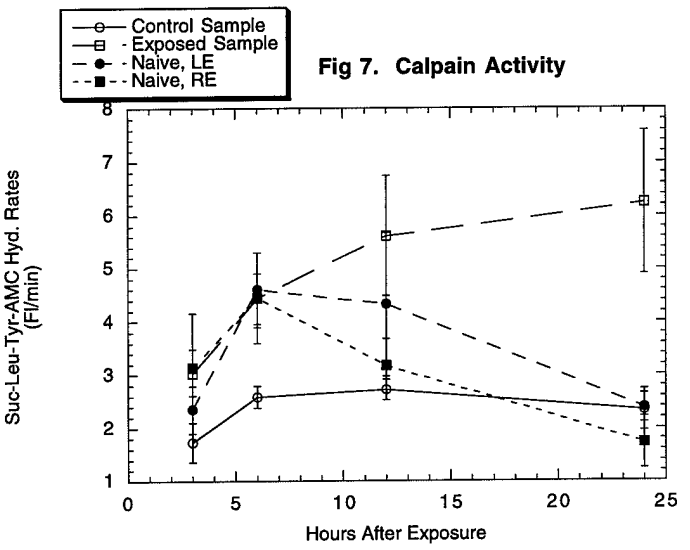


Fig 7. Calpain Activity

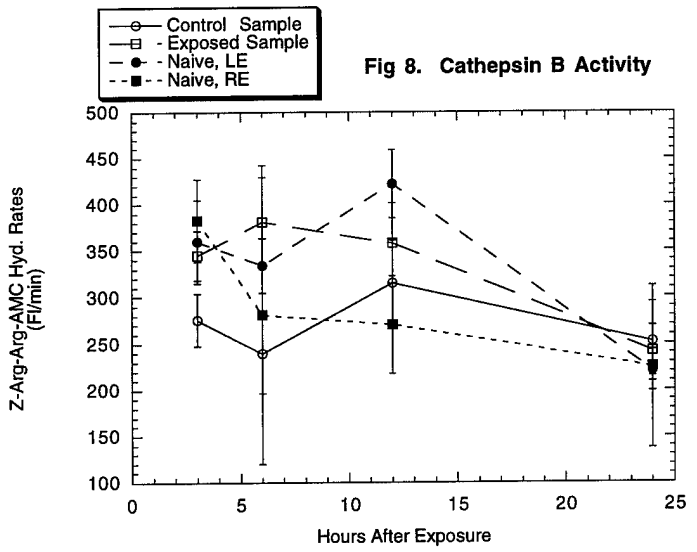


Fig 8. Cathepsin B Activity

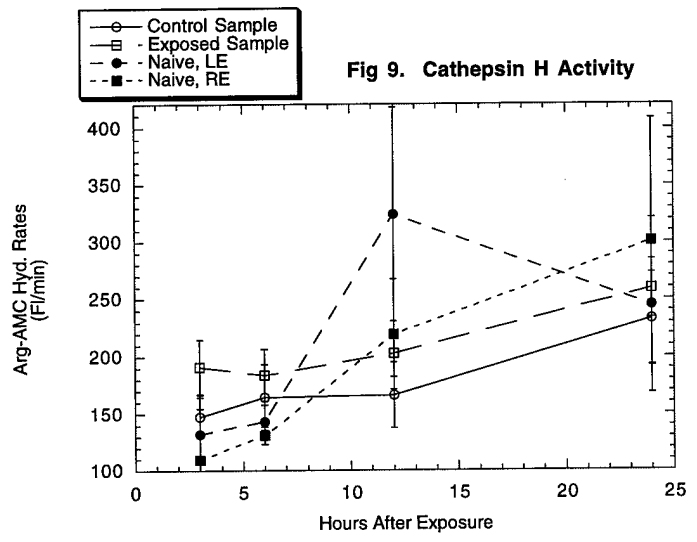


Fig 9. Cathepsin H Activity

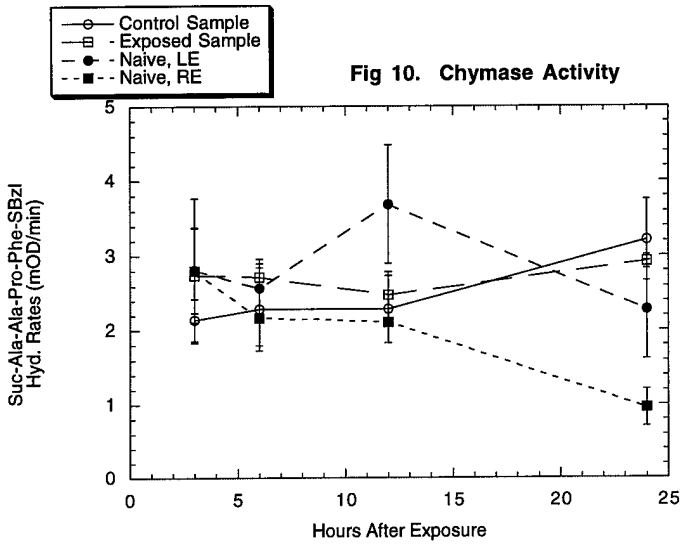


Fig 10. Chymase Activity

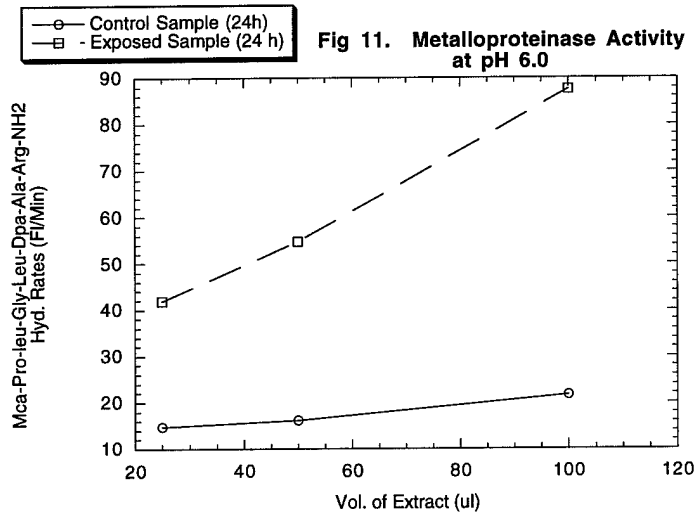
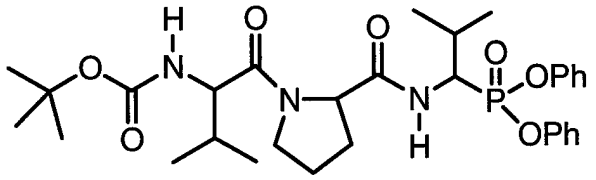
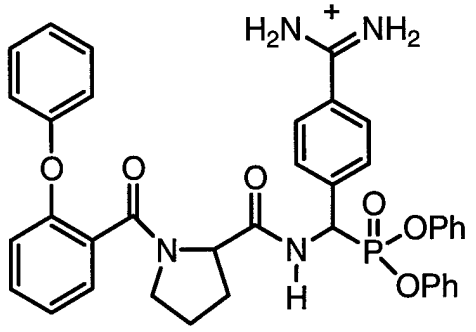


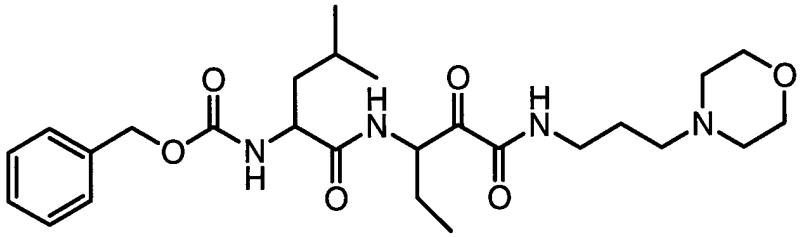
Fig 11. Metalloproteinase Activity at pH 6.0



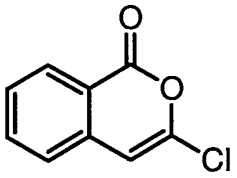
Boc-Val-Pro-Val^{P}(OPh)$_2$



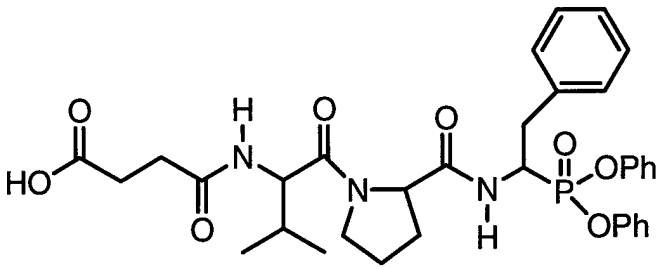
o-phenoxybenzoyl-Pro-4-AmPhGly^{P}(OPh)$_2$



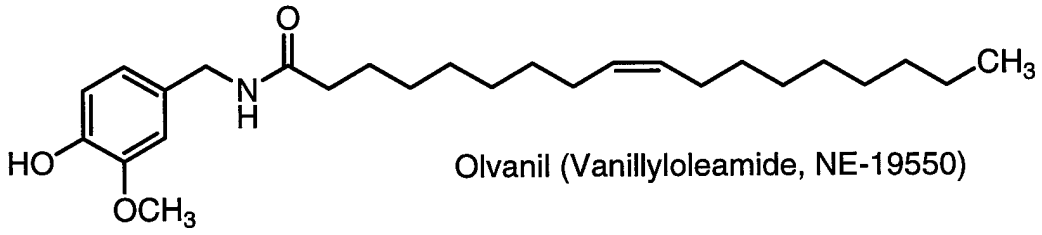
Z-Leu-Abu-CONH-(CH$_2$)$_3$-4-morpholinyl



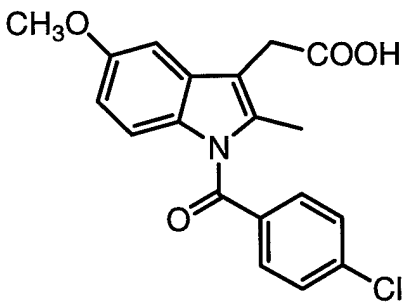
3-Chloroisocoumarin



Suc-Val-Pro-Phe^{P}(OPh)$_2$



Olvanil (Vanillyloleamide, NE-19550)



Indomethacin

Fig 12. Structures of Synthetic Protease Inhibitors and Antiinflammatory Drugs.

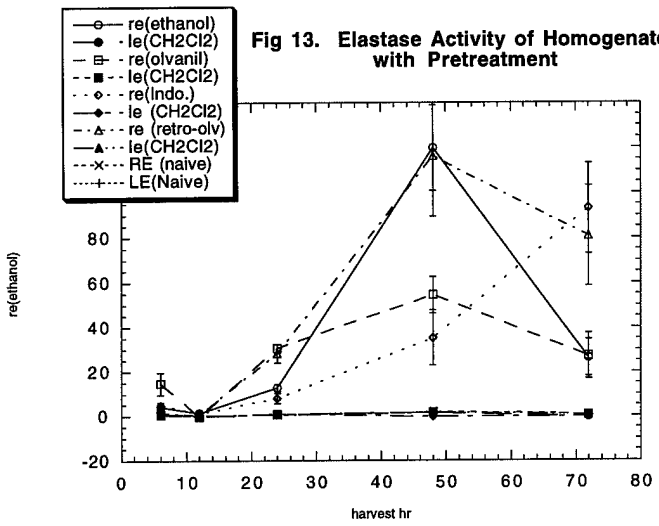


Fig 13. Elastase Activity of Homogenates with Pretreatment

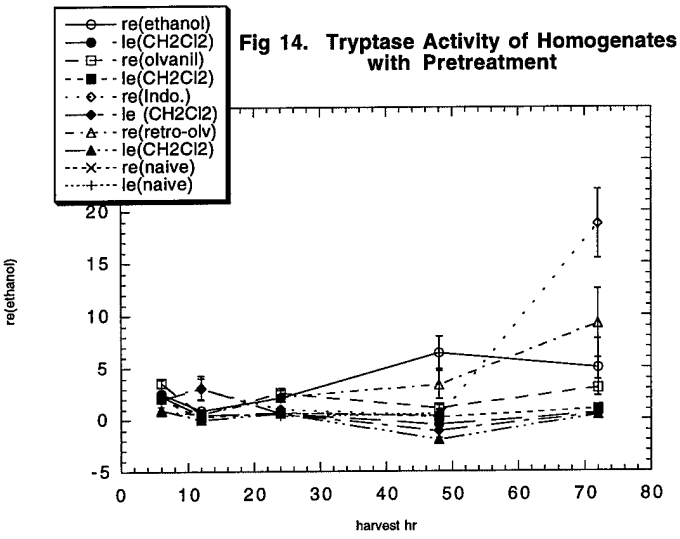


Fig 14. Tryptase Activity of Homogenates with Pretreatment

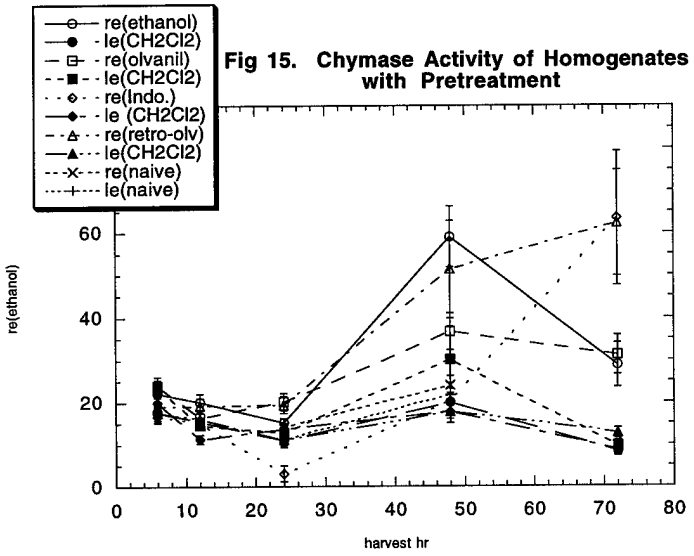


Fig 15. Chymase Activity of Homogenates with Pretreatment

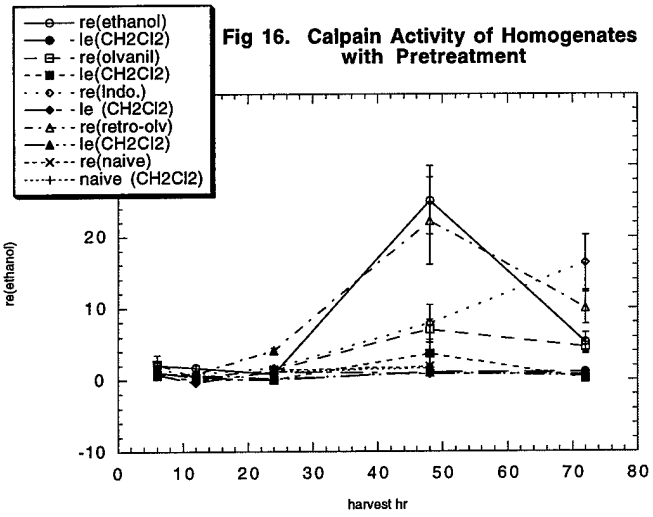


Fig 16. Calpain Activity of Homogenates with Pretreatment

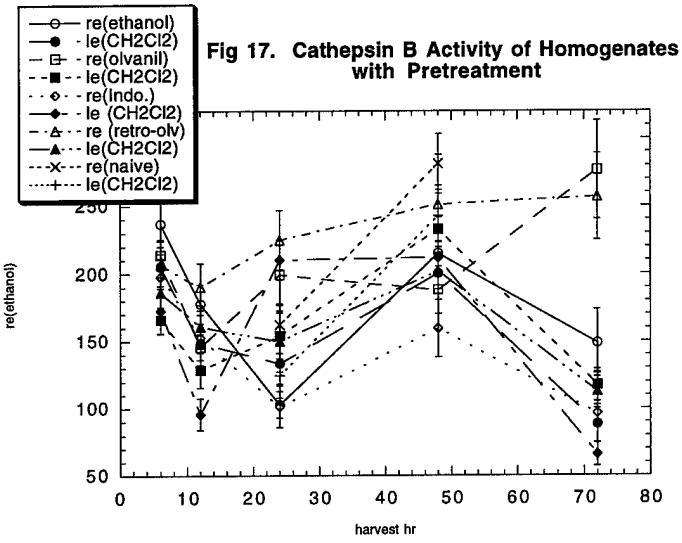


Fig 17. Cathepsin B Activity of Homogenates with Pretreatment

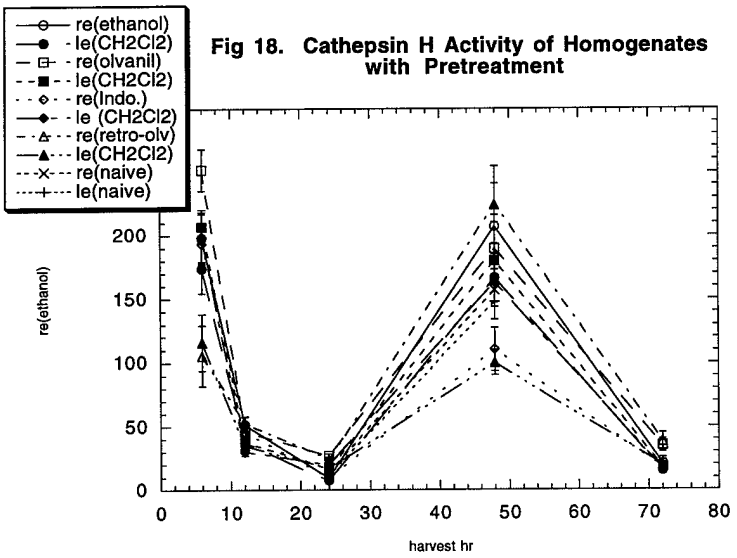


Fig 18. Cathepsin H Activity of Homogenates with Pretreatment

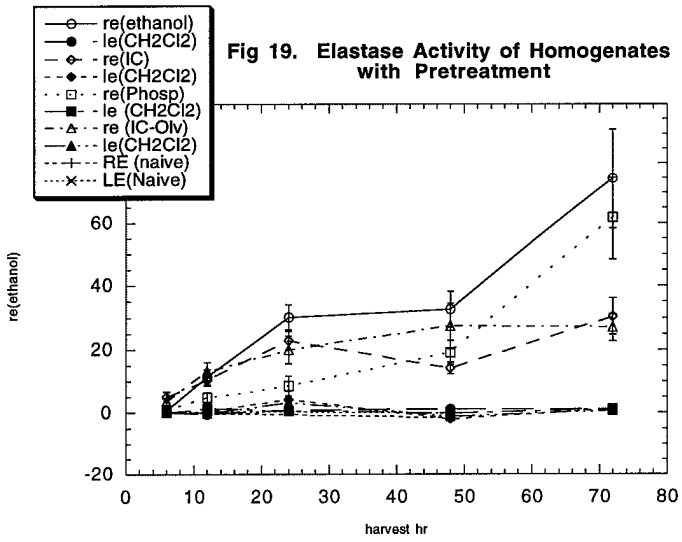


Fig 19. Elastase Activity of Homogenates with Pretreatment

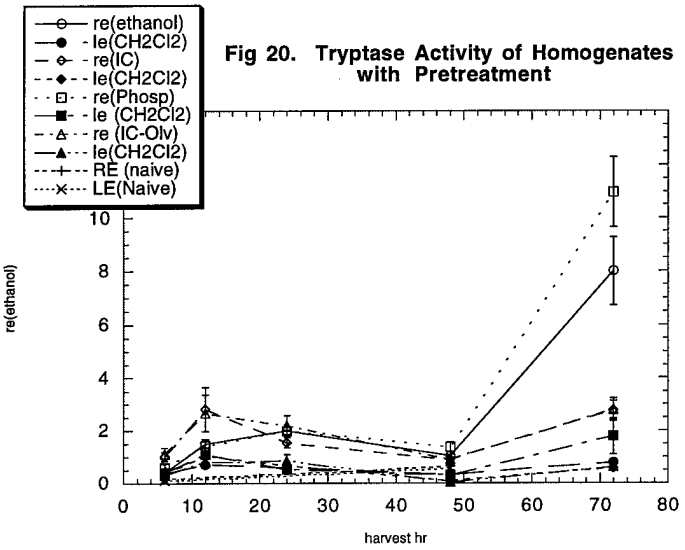


Fig 20. Tryptase Activity of Homogenates with Pretreatment

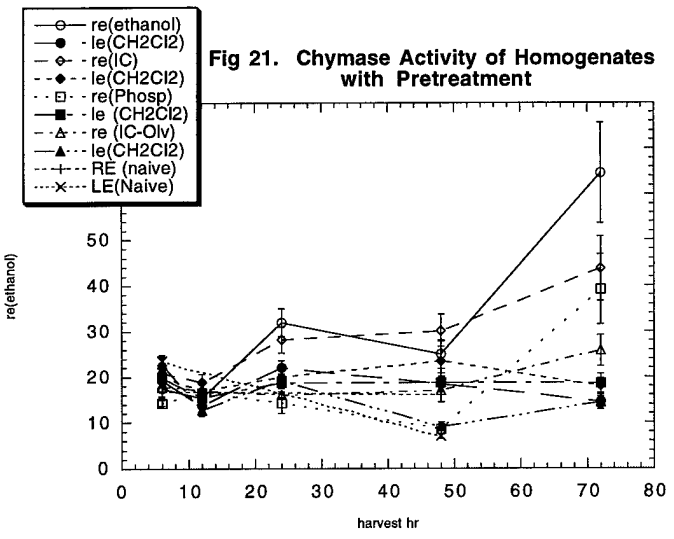


Fig 21. Chymase Activity of Homogenates with Pretreatment

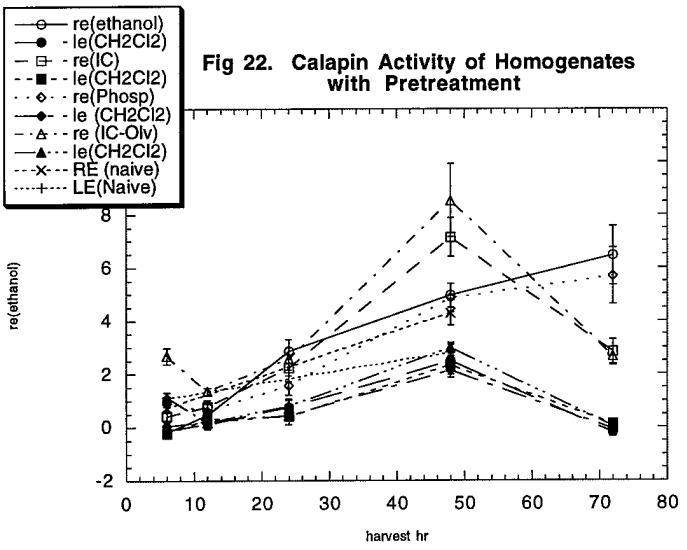


Fig 22. Calapin Activity of Homogenates with Pretreatment

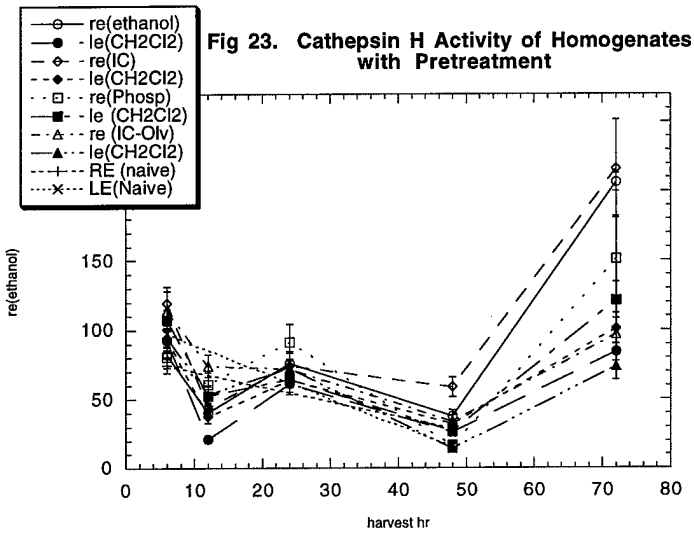


Fig 23. Cathepsin H Activity of Homogenates with Pretreatment

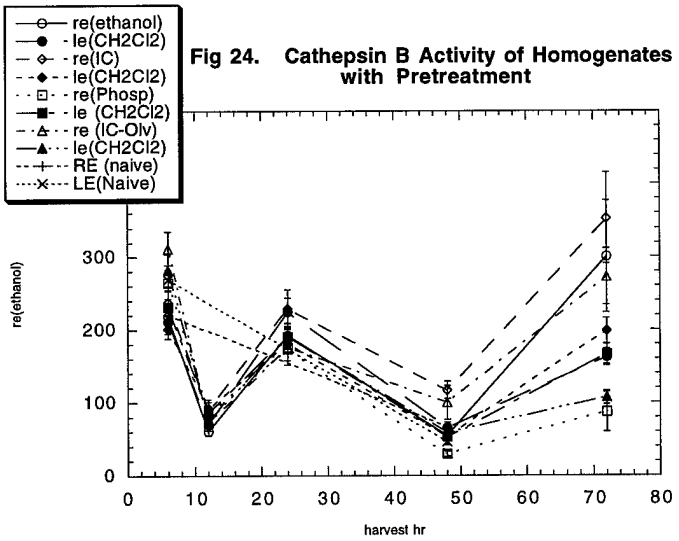


Fig 24. Cathepsin B Activity of Homogenates with Pretreatment

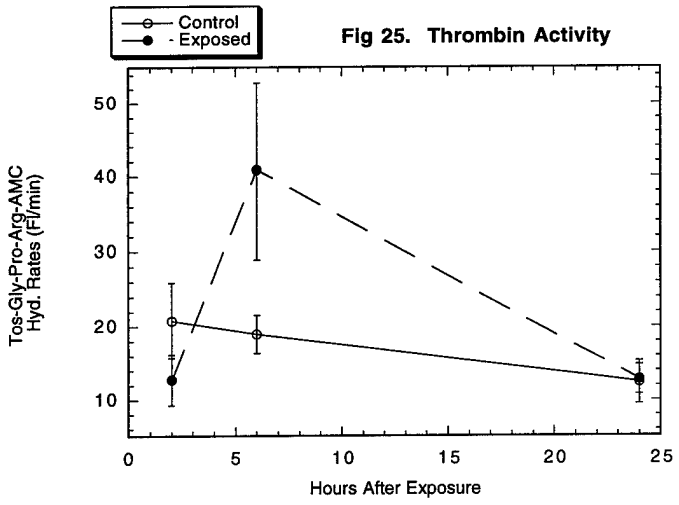


Fig 25. Thrombin Activity

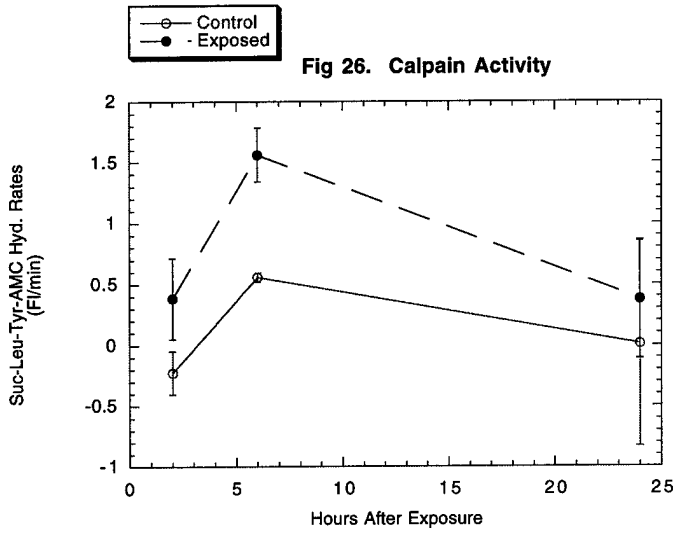


Fig 26. Calpain Activity

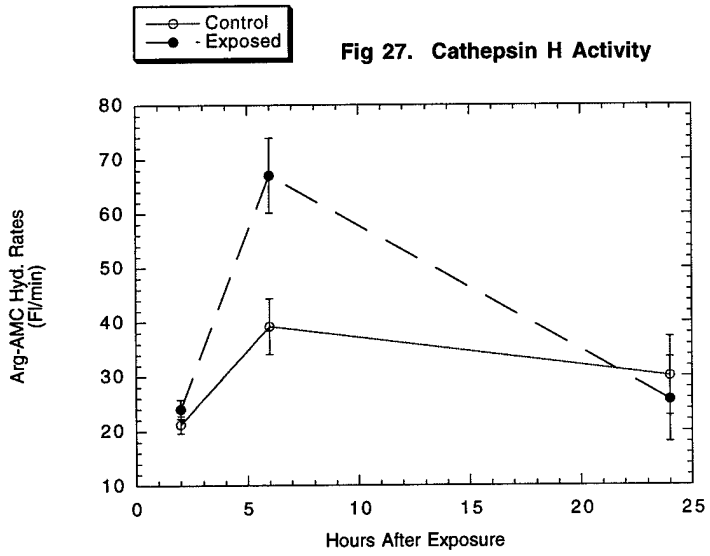


Fig 27. Cathepsin H Activity

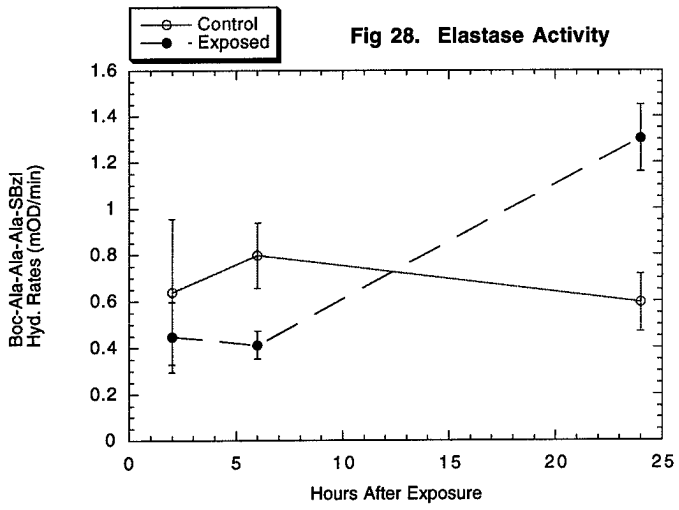


Fig 28. Elastase Activity

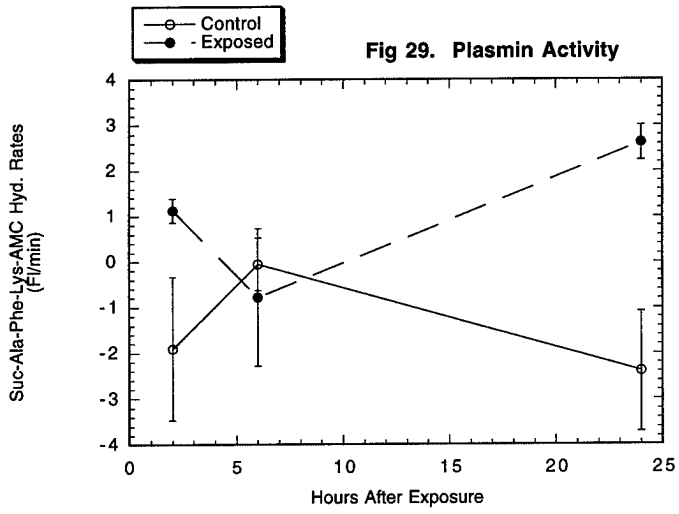


Fig 29. Plasmin Activity

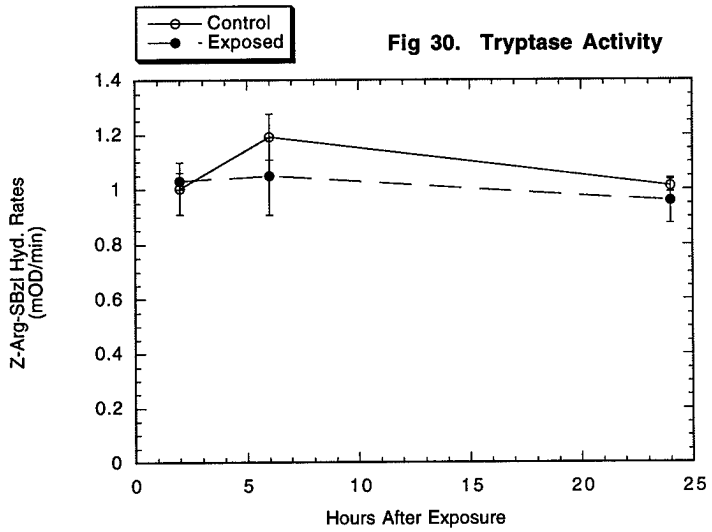


Fig 30. Tryptase Activity

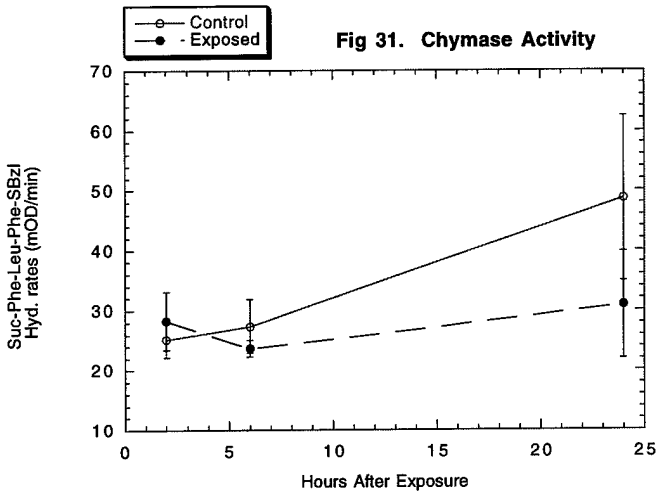


Fig 31. Chymase Activity

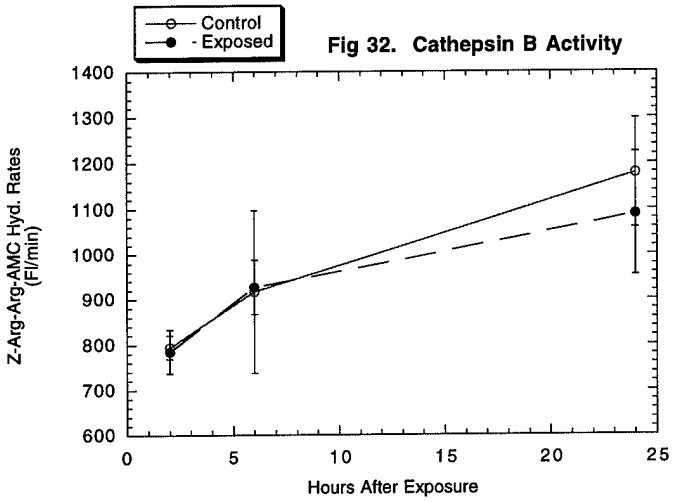


Fig 32. Cathepsin B Activity

APPENDICES

Tables 1-2. Protease activities of the second and third suspensions of skin homogenates from mouse ear exposed to SM.

Tables 3-5. Protease activities of mouse ear skin homogenates pretreated with antiinflammatory drugs at 12, 24, and 72 h postexposure.

Tables 6. Protease activities of mouse ear skin homogenates pretreated with antiinflammatory drugs at 48 h postexposure were effected by the strorage time.

Tables 7-9. Protease activities of mouse ear skin homogenates pretreated with protease inhibitors at 12, 24, and 48 h postexposure.

Table 1. Protease Activities of Mouse Ear Skin Homogenates (Second Suspension).[a]

Hours Postexposure	Enzyme Activity (mOD/min or Fl/min)		
	Exposed Samples	Control Samples	Naive Control
Elastase			
3	-0.02 ± 0.01	-0.03 ± 0.01	0.03 ± 0.03
6	-0.01 ± 0.04	-0.03 ± 0.01	0.12 ± 0.07
12	-0.03 ± 0.01	-0.04 ± 0.02	0.09 ± 0.02
24	0.07 ± 0.01	-0.02 ± 0.01	0.05 ± 0.05
Tryptase (Z-Arg-SBzl)			
3	0.22 ± 0.08	0.14 ± 0.02	0.41 ± 0.08
6	0.54 ± 0.30	0.40 ± 0.33	0.31 ± 0.21
12	0.09 ± 0.08	0.39 ± 0.14	0.23 ± 0.06
24	0.50 ± 0.05	0.13 ± 0.13	0.26 ± 0.12
(Z-Arg-AMC)			
3	0.92 ± 0.74	1.80 ± 0.97	0.13 ± 0.23
6	1.96 ± 0.90	2.97 ± 1.11	1.01 ± 0.26
12	1.36 ± 0.74	3.06 ± 0.34	0.95 ± 0.21
24	2.36 ± 0.23	2.98 ± 1.02	1.36 ± 0.36
Chymase (Suc-Ala-Ala-Pro-Phe-SBzl)			
3	0.33 ± 0.06	0.28 ± 0.02	1.19 ± 0.34
6	0.67 ± 0.15	0.31 ± 0.15	0.66 ± 0.31
12	0.44 ± 0.10	1.05 ± 0.15	1.04 ± 0.33
24	0.58 ± 0.15	0.99 ± 0.30	1.18 ± 1.10
(Suc-Ala-Ala-Pro-Phe-AMC)			
3	-3.30 ± 1.24	0.88 ± 1.18	-2.44 ± 1.74
6	-2.06 ± 0.73	1.71 ± 0.23	-3.12 ± 1.89
12	-2.18 ± 0.47	-0.95 ± 0.10	-2.19 ± 0.35
24	-4.08 ± 0.65	-3.04 ± 1.50	-4.61 ± 1.91
Plasmin			
3	-4.40 ± 0.52	-2.45 ± 0.69	-1.58 ± 0.86
6	-3.45 ± 0.20	-1.52 ± 1.25	-4.13 ± 1.14
12	-3.48 ± 0.23	-1.41 ± 0.35	-4.90 ± 0.96
24	-0.42 ± 0.35	-1.20 ± 1.43	-2.80 ± 1.00
Thrombin			
3	3.76 ± 0.20	5.54 ± 0.19	16.33 ± 10.62
6	8.42 ± 2.62	5.12 ± 2.43	16.37 ± 18.08
12	10.20 ± 1.74	24.50 ± 16.1	11.21 ± 5.17
24	28.58 ± 5.50	5.52 ± 2.54	10.85 ± 12.47
Cathepsin B			
3	58.41 ± 7.82	51.53 ± 3.05	74.65 ± 28.20
6	131.0 ± 26.79	57.13 ± 15.20	71.68 ± 45.03
12	100.9 ± 25.34	112.3 ± 43.33	74.86 ± 9.21
24	35.12 ± 28.93	65.93 ± 19.51	101.3 ± 97.23

Calpain			
3	3.77 ± 0.07	2.98 ± 0.24	2.47 ± 0.30
6	3.10 ± 0.23	5.24 ± 1.46	4.81 ± 0.51
12	2.86 ± 0.17	1.92 ± 0.15	3.62 ± 0.22
24	1.61 ± 0.15	2.97 ± 0.39	3.65 ± 0.71
Cathepsin H			
3	9.65 ± 4.17	11.24 ± 7.47	27.72 ± 15.71
6	31.53 ± 3.07	14.05 ± 14.97	45.88 ± 35.97
12	45.57 ± 13.91	143.1 ± 33.37	96.21 ± 36.29
24	60.24 ± 8.555	34.30 ± 12.30	93.71 ± 77.84

[a]Conditions are described in Materials and Methods.

Table 2. Enzymatic Activities of Mouse Ear Skin Homogenates (Third Suspension).[a]

Hours Postexposure	Enzyme Activity (mOD/min or Fl/min)	
	Exposed Samples	Control Samples
Elastase		
3	-0.04 ± 0.01	-0.04 ± 0.02
6	-0.02 ± 0.03	-0.03 ± 0.01
12	-0.01 ± 0.02	-0.05 ± 0.02
24	0.04 ± 0.02	-0.03 ± 0.01
Tryptase (Z-Arg-SBzl)		
3	0.29 ± 0.16	0.08 ± 0.04
6	0.45 ± 0.35	0.23 ± 0.04
12	0.20 ± 0.04	0.07 ± 0.05
24	0.34 ± 0.15	0.10 ± 0.02
(Z-Arg-AMC)		
3	0.41 ± 0.81	1.03 ± 0.48
6	1.54 ± 0.98	2.88 ± 0.45
12	1.15 ± 1.14	2.63 ± 0.38
24	2.08 ± 0.13	2.14 ± 0.56
Chymase (Suc-Ala-Ala-Pro-Phe-SBzl)		
3	0.21 ± 0.13	0.26 ± 0.06
6	0.38 ± 0.11	0.33 ± 0.03
12	0.42 ± 0.12	0.36 ± 0.00
24	0.60 ± 0.07	0.53 ± 0.04
(Suc-Ala-Ala-Pro-Phe-AMC)		
3	-2.11 ± 0.53	-0.70 ± 0.53
6	-5.27 ± 0.90	-5.21 ± 1.67
12	-2.69 ± 0.60	-2.43 ± 1.19
24	-3.58 ± 1.11	-5.51 ± 1.78
Plasmin		
3	-3.85 ± 0.01	-3.98 ± 0.83
6	-3.93 ± 0.13	-4.44 ± 0.51
12	-3.95 ± 0.20	-4.53 ± 0.88
24	-0.60 ± 0.18	-1.92 ± 0.05
Thrombin		
3	3.03 ± 0.23	3.28 ± 0.23
6	3.92 ± 0.84	7.31 ± 1.03
12	7.36 ± 0.61	10.71 ± 6.71
24	21.72 ± 11.32	3.17 ± 0.81
Cathepsin B		
3	55.94 ± 3.48	22.34 ± 12.99
6	53.64 ± 8.98	55.65 ± 5.81
12	73.82 ± 3.95	67.04 ± 37.12
24	79.41 ± 19.3	24.83 ± 10.82

Calpain		
3	3.80 ± 0.11	3.41 ± 0.64
6	4.86 ± 0.16	5.14 ± 1.11
12	2.33 ± 0.39	4.25 ± 0.09
24	2.22 ± 0.49	4.34 ± 1.12
Cathepsin H		
3	0.26 ± 1.55	-0.35 ± 1.85
6	5.04 ± 4.34	4.90 ± 1.47
12	27.27 ± 7.40	17.87 ± 12.68
24	25.90 ± 16.84	6.74 ± 8.54

[a]Conditions are described in Materials and Methods.

Table 3. Enzymatic Activities of Pretreated Mouse Ear Skin Homogenates at 12 h Postexposure.

Enzyme (Substrate)	Enzyme Activity (mOD/min or Fl/min)	
Pretreatment	Exposed Samples	Control Samples
Elastase		
ethanol	1.27 ± 0.53	0.08 ± 0.07
olvanil	-0.22 ± 0.06	-0.14 ± 0.20
indomethacin	1.54 ± 0.25	0.29 ± 0.19
retro-olvanil	0.70 ± 0.35	-0.04 ± 0.10
Tryptase		
ethanol	0.96 ± 0.19	0.55 ± 0.25
olvanil	0.55 ± 0.13	0.26 ± 0.10
indomethacin	3.04 ± 1.01	3.12 ± 1.15
retro-olvanil	0.62 ± 0.16	0.04 ± 0.04
Chymase		
ethanol	20.1 ± 1.88	15.4 ± 0.86
olvanil	16.4 ± 1.12	14.6 ± 1.00
indomethacin	15.1 ± 1.48	11.3 ± 1.03
retro-olvanil	19.2 ± 1.77	16.2 ± 1.92
Calpain		
ethanol	1.66 ± 0.35	0.37 ± 0.21
olvanil	0.36 ± 0.20	0.21 ± 0.26
indomethacin	0.90 ± 0.23	-0.34 ± 0.14
retro-olvanil	0.90 ± 0.31	0.62 ± 0.30
Cathepsin B		
ethanol	177 ± 14.7	149 ± 6.98
olvanil	146 ± 12.8	129 ± 13.0
indomethacin	153 ± 16.6	95.7 ± 11.8
retro-olvanil	190 ± 17.5	161 ± 15.9
Cathepsin H		
ethanol	51.9 ± 5.9	37.6 ± 2.07
olvanil	46.1 ± 3.35	38.4 ± 3.53
indomethacin	45.7 ± 4.60	30.1 ± 3.12
retro-olvanil	52.5 ± 5.32	35.4 ± 3.77

Table 4. Enzymatic Activities of Pretreated Mouse Ear Skin Homogenates at 24 h Postexposure.

Enzyme (Substrate) In Pretreatment	Enzyme Activity (mOD/min or Fl/min) Exposed Samples	Control Samples	Naive Control
Elastase			
Ethanol	12.7 ± 1.65	0.81 ± 0.12	0.76 ± 0.06
olvanil	30.5 ± 1.83	0.92 ± 0.10	
indomethacin	28.1 ± 4.26	0.88 ± 0.13	
retro-olvanil	7.96 ± 2.35	0.71 ± 0.06	
Tryptase			
ethanol	2.14 ± 0.30	0.67 ±0.09	0.52 ± 0.05
olvanil	2.66 ± 0.21	0.70 ± 0.12	
indomethacin	2.20 ± 0.16	0.60 ± 0.04	
retro-olvanil	1.08 ± 0.17	0.60 ± 0.04	
Chymase			
ethanol	15.1 ± 1.05	11.0 ± 1.62	12.6 ± 0.94
olvanil	20.1 ± 2.06	12.8 ± 1.94	
indomethacin	19.4 ± 2.02	11.1 ± 1.24	
retro-olvanil	3.12 ± 1.87	13.6 ± 1.28	
Calpain			
ethanol	0.81 ± 0.20	0.00 ± 0.14	1.26 ± 0.12
olvanil	1.34 ± 0.15	0.03 ± 0.17	
indomethacin	4.08 ± 0.34	0.14 ± 0.13	
retro-olvanil	1.56 ± 0.32	1.14 ± 0.20	
Cathepsin B			
ethanol	103 ± 9.76	134 ± 15.0	143 ± 15.3
olvanil	199 ± 21.5	154 ± 16.9	
indomethacin	225 ± 22.0	150 ± 22.6	
retro-olvanil	102 ± 15.7	210 ± 14.5	
Cathepsin H			
ethanol	9.97 ± 0.91	7.88 ± 0.65	13.1 ± 1.33
olvanil	27.0 ± 1.72	16.8 ± 2.52	
indomethacin	24.1 ± 2.21	17.3 ± 3.65	
retro-olvanil	9.79 ± 1.63	20.1 ± 2.20	

Table 5. Enzymatic Activities of Pretreated Mouse Ear Skin Homogenates at 72 h Postexposure.

Enzyme (Substrate)	Enzyme Activity (mOD/min or Fl/min)	
Pretreatment	Exposed Samples	Control Samples
Elastase		
ethanol	26.3 ± 8.26	-0.24 ± 0.16
olvanil	27.1 ± 10.3	0.68 ± 0.06
indomethacin	92.9 ± 20.2	-0.24 ± 0.09
retro-olvanil	80.7 ± 22.2	0.48 ± 0.16
Tryptase		
ethanol	5.03 ± 2.72	0.62 ± 0.07
olvanil	3.09 ± 0.76	1.09 ± 0.28
indomethacin	18.8 ± 3.31	0.51 ± 0.10
retro-olvanil	9.22 ± 3.30	0.45 ± 0.03
Chymase		
ethanol	28.78 ± 5.28	8.33 ± 1.05
olvanil	31.2 ± 4.61	9.68 ± 1.32
indomethacin	63.4 ± 16.0	8.80 ± 0.93
retro-olvanil	62.2 ± 12.5	12.6 ± 1.22
Calpain		
ethanol	5.28 ± 1.33	1.03 ± 0.17
olvanil	4.65 ± 1.00	0.14 ± 0.22
indomethacin	16.4 ± 3.89	0.72 ± 0.15
retro-olvanil	9.96 ± 2.23	0.50 ± 0.39
Cathepsin B		
ethanol	149 ± 25.0	88.3 ± 14.0
olvanil	277 ± 36.3	117 ± 11.9
indomethacin	96.0 ± 30.8	65.9 ± 8.63
retro-olvanil	257 ± 32.0	113 ± 13.0
Cathepsin H		
ethanol	20.5 ± 5.10	15.4 ± 2.23
olvanil	35.2 ± 4.97	18.2 ± 1.39
indomethacin	18.2 ± 5.46	16.5 ± 1.24
retro-olvanil	38.4 ± 6.60	19.8 ± 1.64

Table 6. Protease Activities of Exposed Samples Pretreated with Antiinflammatory Drugs at 48 h Postexposure Were Effected by the Strorage Time.

Enzyme	Enzyme Activity (mOD/min or Fl/min)	
Pretreatment	Before Storage	After Storage at -20 °C
Elastase		
ethanol	120	43.5
olvanil	54.4	25.4
indomethacin	35.2	16.3
retro-olvanil	116	58.9
Tryptase		
ethanol	6.45	9.02
olvanil	1.15	6.43
indomethacin	0.40	4.02
retro-olvanil	3.39	13.0
Chymase		
ethanol	59.0	45.4
olvanil	36.7	25.7
indomethacin	20.3	14.0
retro-olvanil	51.3	43.5
Calpain		
ethanol	25.1	5.20
olvanil	7.01	4.22
indomethacin	7.81	4.43
retro-olvanil	22.2	8.21
Cathepsin B		
ethanol	215	253
olvanil	188	231
indomethacin	159	118
retro-olvanil	251	351
Cathepsin H		
ethanol	207	15.8
olvanil	190	56.8
indomethacin	110	12.6
retro-olvanil	224	45.0

Table 7. Enzymatic Activities of Pretreated Mouse Ear Skin Homogenates at 12 h Postexposure.

Enzyme (Substrate) In Pretreatment	Enzyme Activity (mOD/min or Fl/min) Exposed Samples	Control Samples
Elastase (Boc-Ala-Ala-Ala-SBzl)		
Ethanol	11.4 ± 2.42	-0.41 ± 0.22
3-chloroisocoumarin	10.4 ± 1.84	0.26 ± 0.10
Suc-Val-Pro-Phe^{P}(OPh)$_2$	4.83 ± 1.79	1.30 ± 0.89
3-chloroisocoumarin + olvanil	13.2 ± 2.79	-0.12 ± 0.18
Tryptase		
ethanol	1.51 ± 0.18	0.73 ± 0.06
3-chloroisocoumarin	2.82 ± 0.85	1.04 ± 0.12
Suc-Val-Pro-Phe^{P}(OPh)$_2$	1.42 ± 0.22	1.09 ± 0.26
3-chloroisocoumarin + olvanil	2.68 ± 0.69	0.82 ± 0.16
Chymase		
ethanol	15.3 ± 1.52	13.7 ± 1.47
3-chloroisocoumarin	18.8 ± 1.80	17.1 ± 1.96
Suc-Val-Pro-Phe^{P}(OPh)$_2$	16.5 ± 2.16	15.5 ± 1.85
3-chloroisocoumarin + olvanil	17.0 ± 1.77	12.6 ± 1.13
Calpain		
ethanol	0.46 ± 0.22	0.14 ± 0.20
3-chloroisocoumarin	0.78 ± 0.24	0.31 ± 0.18
Suc-Val-Pro-Phe^{P}(OPh)$_2$	0.62 ± 0.25	0.23 ± 0.17
3-chloroisocoumarin + olvanil	1.35 ± 0.14	0.17 ± 0.11
Cathepsin B		
ethanol	61.0 ± 6.42	70.4 ± 8.68
3-chloroisocoumarin	91.9 ± 12.7	92.2 ± 8.91
Suc-Val-Pro-Phe^{P}(OPh)$_2$	84.7 ± 10.1	86.2 ± 9.88
3-chloroisocoumarin + olvanil	72.4 ± 7.08	75.2 ± 6.74
Cathepsin H		
ethanol	40.3 ± 7.42	21.1 ± 2.66
3-chloroisocoumarin	43.7 ± 3.40	37.6 ± 4.30
Suc-Val-Pro-Phe^{P}(OPh)$_2$	60.8 ± 8.58	52.5 ± 6.99
3-chloroisocoumarin + olvanil	74.4 ± 8.20	53.3 ± 6.84

Table 8. Enzymatic Activities of Pretreated Mouse Ear Skin Homogenates at 24 h Postexposure.

Enzyme (Substrate) In Pretreatment	Enzyme Activity (mOD/min or Fl/min) Exposed Samples	Control Samples
Elastase (Boc-Ala-Ala-Ala-SBzl)		
Ethanol	30.2 ± 3.97	0.97 ± 0.24
3-chloroisocoumarin	22.8 ± 2.91	4.19 ± 2.73
Suc-Val-Pro-Phe^{P}(OPh)$_2$	8.52 ± 3.12	0.61 ± 0.07
3-chloroisocoumarin + olvanil	19.97 ± 4.37	3.25 ± 2.28
Tryptase		
ethanol	2.0 ± 0.13	0.59 ± 0.03
3-chloroisocoumarin	1.56 ± 0.19	0.67 ± 0.13
Suc-Val-Pro-Phe^{P}(OPh)$_2$	1.98 ± 0.19	0.53 ± 0.03
3-chloroisocoumarin + olvanil	2.20 ± 0.38	0.85 ± 0.26
Chymase (Suc-Phe-Leu-Phe-SBzl)		
ethanol	31.9 ± 3.08	22.0 ± 1.49
3-chloroisocoumarin	28.1 ± 2.85	19.9 ± 1.80
Suc-Val-Pro-Phe^{P}(OPh)$_2$	14.2 ± 2.15	18.7 ± 1.35
Isocoumarin + olvanil	16.1 ± 0.66	19.2 ± 1.45
Calpain		
ethanol	2.85 ± 0.43	0.76 ± 0.30
3-chloroisocoumarin	2.23 ± 0.50	0.41 ± 0.31
Suc-Val-Pro-Phe^{P}(OPh)$_2$	1.57 ± 0.37	0.45 ± 0.16
3-chloroisocoumarin + olvanil	2.57 ± 0.41	0.81 ± 0.21
Cathepsin B		
ethanol	193 ± 17.1	225 ± 19.3
3-chloroisocoumarin	229 ± 26.1	182 ± 23.6
Suc-Val-Pro-Phe^{P}(OPh)$_2$	173 ± 15.3	191 ± 18.2
3-chloroisocoumarin + olvanil	176 ± 25.1	191 ± 10.7
Cathepsin H		
ethanol	76.2 ± 6.96	61.4 ± 6.0
3-chloroisocoumarin	74.4 ± 10.2	64.0 ± 10.5
Suc-Val-Pro-Phe^{P}(OPh)$_2$	91.4 ± 12.5	64.6 ± 6.0
3-chloroisocoumarin + olvanil	71.8 ± 7.61	72.4 ± 5.5

Table 9. Enzymatic Activities of Pretreated Mouse Ear Skin Homogenates at 48 h Postexposure.

Enzyme (Substrate) In Pretreatment	Enzyme Activity (mOD/min or Fl/min)		
	Exposed Samples	Control Samples	Naive Control
Elastase			
Ethanol	32.6 ± 5.56	1.09 ± 0.11	-1.43 ± 0.08
3-chloroisocoumarin	14.2 ± 1.88	-2.01 ± 0.13	
Suc-Val-Pro-Phe^{P}(OPh)$_2$	19.1 ± 3.74	-0.11 ± 0.12	
3-chloroisocoumarin + olvanil	27.4 ± 7.06	-1.12 ± 0.11	
Tryptase			
ethanol	1.05 ± 0.13	0.33 ± 0.17	0.61 ± 0.14
3-chloroisocoumarin	0.92 ± 0.06	0.09 ± 0.04	
Suc-Val-Pro-Phe^{P}(OPh)$_2$	1.38 ± 0.19	0.31 ± 0.06	
3-chloroisocoumarin + olvanil	0.91 ± 0.19	0.04 ± 0.03	
Chymase			
ethanol	24.9 ± 3.09	18.4 ± 1.79	1.15 ± 1.0
3-chloroisocoumarin	30.2 ± 3.52	23.4 ± 4.39	
Suc-Val-Pro-Phe^{P}(OPh)$_2$	8.55 ± 1.14	18.7 ± 2.07	
3-chloroisocoumarin + olvanil	17.1 ± 2.75	9.10 ± 0.93	
Calpain			
ethanol	4.95 ± 0.45	2.50 ± 0.20	3.55 ± 0.38
3-chloroisocoumarin	7.14 ± 0.73	2.33 ± 0.33	
Suc-Val-Pro-Phe^{P}(OPh)$_2$	4.86 ± 0.54	2.12 ± 0.26	
Isocoumarin + olvanil	8.54 ± 1.38	2.97 ± 0.20	
Cathepsin B			
ethanol	55.4 ± 5.94	65.1 ± 5.81	56.7 ± 5.33
3-chloroisocoumarin	117 ± 11.7	53.2 ± 5.72	
Suc-Val-Pro-Phe^{P}(OPh)$_2$	29.6 ± 3.53	53.3 ± 5.11	
3-chloroisocoumarin + olvanil	100 ± 23.3	59.5 ± 5.96	
Cathepsin H			
ethanol	37.3 ± 4.51	26.1 ± 3.14	22.6 ± 2.27
3-chloroisocoumarin	60 ± 7.21	32.1 ± 3.06	
Suc-Val-Pro-Phe^{P}(OPh)$_2$	16.8 ± 2.26	26.8 ± 3.50	
3-chloroisocoumarin + olvanil	34.1 ± 4.51	14.3 ± 1.12	